

**YB-1 IS A DOWNSTREAM COMPONENT OF THE PI3K/AKT SIGNALING PATHWAY
AND REGULATES EGFR IN BREAST CARCINOMA:
A MECHANISM FOR BREAST CANCER GROWTH**

by

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ABSTRACT

High level of activated or phosphorylated serine/threonine kinase Akt is commonly observed in aggressive breast cancer. Hence, the Akt signaling pathway has become a popular target for therapeutic interventions. Previously our lab discovered that phosphorylated Akt is co-expressed with a transcription/translation factor called the Y-box Binding Protein-1 (YB-1) and that Akt phosphorylates YB-1 at Ser102 *in vitro*. It was also found that YB-1, but not the YB-1 mutant in which Ser102 is mutated to alanine (YB-1A102), enhanced breast cancer cell growth. Since YB-1 was originally isolated as a DNA binding protein that interacts with the regulatory elements of the epidermal growth factor receptor (*EGFR*), we addressed the possibility that phosphorylated YB-1 stimulates breast cancer cell growth by up-regulating *EGFR* expression. First, we demonstrated that YB-1 was phosphorylated through the PI3K/Akt pathway *ex vivo* by immunoprecipitation and western blotting. We then illustrated that loss of Ser102 affects the nuclear translocation of YB-1, implying that Akt may also regulate YB-1 nuclear trafficking. By exogenously expressing YB-1 or YB-1A102 in breast cancer cell lines we showed that YB-1 but not YB-1A102 induced the levels of *EGFR* mRNA and protein. It was revealed that YB-1 bound to the -2 kb of the *EGFR* promoter by chromatin immunoprecipitation. Interestingly, disruption of Ser102 prevented YB-1 from interacting with the first 1 kb of the *EGFR* promoter, indicating that binding to this region by YB-1 is regulated by the PI3K/Akt signaling. We then demonstrated that mutation of Ser102 perturbed YB-1 from activating the -1 kb of the *EGFR* promoter by luciferase reporter assays. Finally, since activation of Akt depends on 3-phosphoinositide-dependent protein kinase-1 (PDK1), we examined the potential of a PDK1 inhibitor, OSU-03012, to inhibit the functions of YB-1. It was found that OSU-03012

blocked YB-1 nuclear translocation and binding to the -1 kb promoter of *EGFR*. Together these results suggested that activation of the PI3K/Akt pathway leads to phosphorylation of YB-1 at Ser102, resulting in up-regulation of *EGFR* gene expression. Here we propose that Akt, YB-1, and EGFR may all function in the same pathway to promote breast cancer cell growth.

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ABBREVIATIONS

AF-1	Activation function-1
AF-2	Activation function-2
AFX	ALL1 fused gene from chromosome X
AI	Aromatase inhibitor
BAD	BCL2-antagonist of cell death
CDK	Cyclin-cyclin-dependent kinase
COX-2	Cyclooxygenase-2
CRS	Cytoplasmic retention site
CSD	Cold shock domain
CTD	C-terminal domain
DbpA	DNA binding protein A
DbpB	DNA binding protein B
DbpC	DNA binding protein C
DMSO	Dimethyl Sulphonite
DNA-PK	DNA-dependent protein kinase
4E-BP1	4E-binding protein 1
EGF	Epidermal growth factor
Egf-1	Early growth response factor-1
EGFR	Epidermal growth factor receptor
ELB	Egg lysis buffer
ER	Estrogen receptor

ERDBP-1	EGF-responsive DNA-binding protein-1
ERK1	Extracellular-signal-regulated kinase 1
ES cell	Embryonic stem cell
ETR	EGFR transcriptional repressor
FBS	Fetal bovine serum
FKHR	Forkhead in rhabdomyosarcoma
GCF	GC factor
GSK3	Glycogen synthase kinase 3
GSK3 β	Glycogen synthase kinase-3 β
HB-EGF	Heparin-binding EGF
HIF-1 α	Hypoxia inducible factor-1
HIV-I	Human T-cell lymphotropic virus type I
HTLV-I	Human T-cell lymphotropic virus type I
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IKK	I κ B kinase
ILK	Integrin-linked kinase
INF- γ	Interferon γ
Jak	IFN- γ -Janus kinase
JCV	JC virus
LARII	Luciferase assay reagent II
MAPK	Mitogen-activated protein kinase
MDR-1	Multidrug resistance 1
MHC	Major histocompatibility complex

MMP-2	Matrix metalloproteinase 2
MMP-12	Matrix metalloproteinase 12
mRNP	Messenger ribonucleoprotein particles
MRP 1	Multidrug resistance-related protein 1
MSH2	MutS homologue 2
MVP	Major vault protein
NF- κ B	Nuclear factor kappa B
NLS	Nuclear localization signal
NSCLC	Non-small-cell lung cancer
NSEP-1	Nuclease-sensitive element protein-1
pAkt	Phosphorylated Akt
PCNA	Proliferating cell nuclear antigen
PDK1	3-Phosphoinositide-dependent protein kinase-1
PH	Pleckstrin-homology
PIC	Phosphatase inhibitor cocktail
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKB	Protein kinase B
PKC- ϵ	Protein kinase C-epsilon
PLB	Passive lysis buffer
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
pSer-YB-1	Phospho-Ser-YB-1

PTEN	Phosphatase and tensin homologue
RIPA	Radio-immunoprecipitation assay
RSK	p70 Ribosomal S6 kinase
RSV-d	Rous sarcoma virus d
RTK	Receptor tyrosine kinase
SERM	Selective estrogen receptor modulator
SH2	SRC-homology-2
TAE	Tris-acetate-EDTA
TBP	TATA-binding protein
TGF- α	Transforming growth factor- α
TGF- β	Transforming growth factor- β
TMA	Tumor tissue microarray
VEGF	Vascular endothelial growth factor
WRN	Werner syndrome protein
YB-1	Y-box binding protein-1

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DEDICATION

To my family,

Eric Wu, Janet Lee, and Jelemee Wu

For all their love and support

CHAPTER 1

INTRODUCTION

Cancers develop through a multi-step process, which reflects the genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg 2000). In the United States a total of 556,902 cancer deaths were recorded in 2003 (Jemal *et. al.* 2006). A study reported by Jemal *et. al.* also revealed that currently one in four deaths is due to cancer (Jemal *et. al.* 2006). With statistics like these, it is not surprising that cancer has surpassed heart disease as the leading cause of mortality for Americans under age of 85 since 1999 (Jemal *et. al.* 2006).

1.1 Cancer of the Breast

1.1.1 Frequency and Origin

Among women in North America (Jemal *et. al.* 2006, www.cancer.ca), breast carcinoma was estimated to be the most common malignancy and the second leading cause of cancer deaths in women in 2006. This year, 212,920 of American women will be diagnosed with breast cancer and 40,970 will die of it (Jemal *et. al.* 2006). The statistics for Canadian women are also devastating as 22,300 new breast cancer cases will be expected and 5,300 patients will not survive the disease (www.cancer.ca). In these patients, it is not the primary tumor, but metastases at distant sites that are the main cause of deaths. According to the American Cancer Society, patients with localized breast tumors can expect a 98% chance of survival five years post-diagnosis. However, once the cancer has spread to distant sites such as the lungs, liver, or bone, the five-year survival rate drops to as low as 16% (www.cancer.org).

In breast cancer both genetic and environmental factors, such as age and increased hormone exposure, contribute to the accumulation of mutations in essential genes involved in cell cycle check points, cell survival and growth, and DNA repair (Baselga and Norton 2002). The most common genetic abnormalities in both familial and sporadic breast cancers are loss of heterozygosity (LOH) at multiple loci resulting in mutations in alleles of tumor suppressor genes (Baselga and Norton 2002). Loss of heterozygosity in *BRCA1* (Hall *et. al.* 1990, Miki *et. al.* 1994), *BRCA2* (Wooster *et. al.* 1994, Wooster *et. al.* 1995), *P53* (Malkin *et. al.* 1990, Sidransky *et. al.* 1992), and *PTEN* (Liaw *et. al.* 1997) has been linked to hereditary breast tumors. Amplification of oncogenes, including *ERBB2* (Slamon *et. al.* 1987), *C-MYC* (Escot *et. al.* 1986), and *CYCLIN D1* (Buckley *et. al.* 1993), accounts for the second most common genetic alteration in breast carcinoma (Baselga and Norton 2002). In addition, the activation of certain receptors such as the estrogen receptors and the epidermal growth factor receptors is also implicated in the pathogenesis of breast malignancies (section 1.1.2). While 5-10% of all breast cancers in women are hereditary, most breast tumors are sporadic (Mincey 2003).

1.1.2 Classification of Breast Cancer

Distinct subtypes of sporadic breast cancer have been identified using gene-expression microarray analysis. There are two main groups of breast carcinoma, namely the estrogen receptor-positive (ER(+)) and the estrogen receptor-negative (ER(-)) breast cancers (Perou *et. al.* 2000, Sorlie *et. al.* 2001, 2003). The ER(+) tumors are further classified into luminal subtype A and B, depending on their expression levels of cytokeratin-8 and cytokeratin-18. For ER(-) breast carcinoma, three biologically distinct subgroups are identified: 1) normal breast-like, 2) ERBB2+, and 3) basal-like. The normal breast-like tumors are characterized by high levels of many gene products expressed by the adipose tissue and other non-epithelial cell types. The ERBB2+

subgroup over-expresses the ERBB2 protein (also known as Her-2), a member of the epidermal growth factor receptor subfamily, while the basal-like tumors express high levels of cytokeratin-5 and cytokeratin-7. Survival analyses on the subtypes have been conducted in two separate patient cohorts. In one cohort patients were treated with both pre-surgical chemotherapy and post-surgical endocrine therapy (Sorlie *et. al.* 2001) whereas patients from another group (van't Veer *et. al.* 2002) did not receive any systemic adjuvant treatment. Studies on both data sets revealed similar results in which patients with ER(+) tumors have a relatively favorable prognosis (Sorlie *et. al.* 2003). In contrast, the ER(-) ERBB2+ and basal-like subtype breast cancers are invasive and more likely to metastasize, and patients with these types of tumors have the shortest survival times (Sorlie *et. al.* 2003).

1.1.3 Standard Treatment of Breast Cancer

For breast carcinomas that have not metastasized, the first line treatments include surgery and radiation therapies (Baselga and Norton 2002). Most breast cancer patients also receive adjuvant chemotherapies to eradicate residual cancer cells that may have spread to secondary sites (Baselga and Norton 2002, Houssami *et. al.* 2006). The most frequently used chemotherapeutic drugs are alkylating agents (cyclophosphamide), anthracyclins (doxorubicin), antimetabolites (5-fluorouracil), and antimicrotubule agents (paclitaxel and docetaxel) (Baselga and Norton 2002). These agents inhibit cancer growth by interfering with DNA synthesis or mitosis. For example, doxorubicin affects the activity of topoisomerase II, while paclitaxel interferes with microtubule functioning (Baselga and Norton 2002).

Given that DNA and cell replication also occur in normal cells, traditional chemotherapies to cancers are often hindered by toxicity and lack of efficacy. Consequently, there is growing interest in developing treatments that more specifically target tumor cells. Such

treatment, termed targeted therapy, exploits the reliance of malignant cells on continued stimulation by hormones or growth factors as well as constitutive activation of downstream signaling pathways. The essential role of estrogen signaling in breast tumorigenesis led to the development of hormonal therapy. It was known that upon estrogen binding, ER becomes activated and associates with transcription factors such as AP-1 and Sp1 to promote gene expression and cell growth (Sommer *et. al.* 2001, Webb *et. al.* 1999, Porter *et. al.* 1997). One well known example of hormonal therapy is the selective estrogen receptor modulator (SERM) that competes with estrogen to bind to ER, thereby blocking the ligand-dependent activation function 2 (AF-2) domain of ER without inhibiting the activity of the ligand independent activation function 1 (AF-1) domain (Shao and Brown 2004). Currently, tamoxifen is the SERM frequently prescribed for the treatment of all stages of breast cancer (Shao and Brown 2004). Other SERMs, namely toremifen and raloxifene, have also been approved by the Food and Drug Association (FDA) for clinical use in the USA (Shao and Brown 2004). Faslodex, or fulvestrant, is a recently introduced novel "pure ER antagonist" that provides further option in hormonal therapy. Unlike tamoxifen, fulvestrant inhibits both AF-1 and AF-2 domains of ER and has no estrogen agonist activity (Fallowfield 2004, Howell 2005). Lastly, aromatase inhibitors (AIs) are hormonal drugs that act by inhibiting estrogen production in peripheral tissues in postmenopausal women. AIs that have started to be administered to postmenopausal breast cancer patients include anastrozole, letrozole, and exemstane (Fallowfield 2004).

The efficacy of hormonal drugs such as tamoxifen in many breast cancer patients is limited by a few factors. First of all, although tamoxifen was shown to be beneficial to patients with ER(+) breast tumors (EBCT 1998a), approximately 30-40% of the breast tumors are ER(-) (Shao and Brown 2004). Herceptin, a humanized monoclonal antibody targeting ERBB2, was approved for treating ER(-) ERBB2-overexpressing metastatic breast cancers (section 1.2.4).

However other ER(-) breast tumors, such as the aggressive basal-like breast cancer, are still difficult to manage by current therapies. Secondly, almost all patients with advanced breast cancer who initially responded to tamoxifen therapy developed resistance (Shao and Brown 2004). Reasons for tamoxifen resistance include the rare loss and mutation of ER, altered levels of ER co-regulators, and importantly, aberrant activation of additional growth factor signaling pathways (Shao and Brown 2004, Kurokawa and Arteaga 2003). Therefore, there is a need to gain more knowledge on other growth pathways that are deregulated in breast cancer, especially those that are in advanced stages and/or do not express ER, so that novel therapeutic strategies can be developed.

1.2 The PI3K/Akt Signaling Pathway

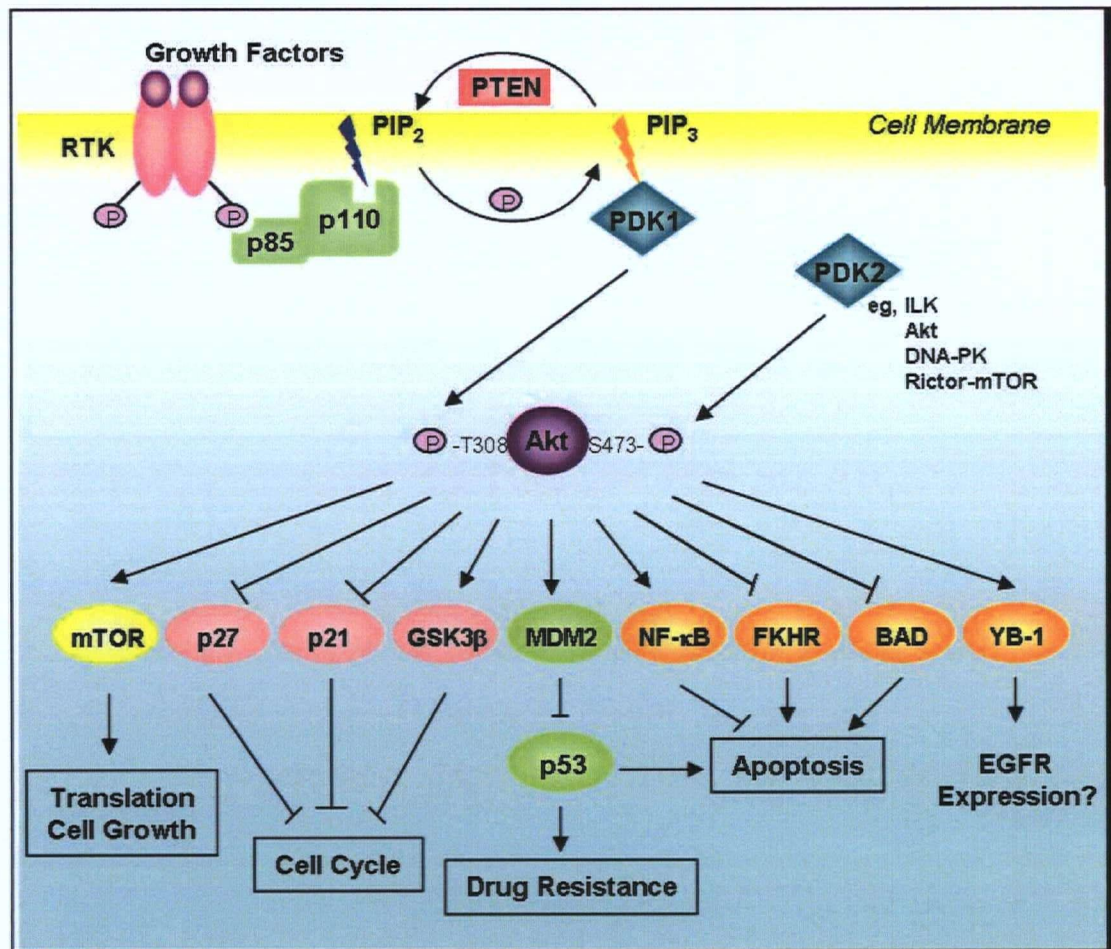
Of the growth factor signaling pathways, the phosphatidylinositol-3 kinase (PI3K)/Akt cascade (Figure 1) has crucial roles in regulating divergent cellular processes including apoptosis, proliferation, differentiation, and metabolism. In recent years, the PI3K/Akt signaling has attracted much attention as components of this pathway are frequently deregulated in a wide spectrum of cancers.

1.2.1 The Signaling Cascade

Phosphatidylinositol-3 kinases (PI3Ks) constitute a lipid kinase family characterized by their ability to phosphorylate the 3'-OH group of inositol ring in inositol phospholipids (Fruman *et. al.* 1998). The PI3K family is divided into three classes (I, II, III) based on structure, substrate preference, and function (Vanhaesebroeck and Waterfield 1999, Cantley *et. al.* 2002, Okkenhaug and Vanhaesebroeck 2003, Wymann *et. al.* 2003, Deane and Fruman 2004). The primary PI3K proteins that respond to growth factors belong to class Ia. The class Ia PI3Ks are

Figure 1
The PI3K/Akt Signaling Pathway

Upon growth factor binding, receptor tyrosine kinases dimerize leading to autophosphorylation and recruitment of PI3K via the p85 subunit to the membrane. The p110 subunit of PI3K then generates PIP3 from PIP2. The tumor suppressor PTEN can convert PIP3 to PIP2 thus inactivate the signaling. PIP3 acts as a second messenger to activate PDK1, which phosphorylates Akt at Thr308. For full activation, Akt is further phosphorylated at Ser473 by ILK, Akt itself, DNA-PK, rictor-mTOR complex, or an unidentified PDK2. The phosphorylated Akt then phosphorylates a wide range of proteins, leading to inhibition of apoptosis, drug resistance, cell cycle progression, translation and cell growth.



heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit (Fruman *et. al.* 1998). Upon binding by growth factors, tyrosine residues of transmembrane receptor tyrosine kinases (RTKs), such as the insulin-like growth factor-1 receptor (IGF-1R) and the epidermal growth factor receptors including ERBB-1 and ERBB-2, become phosphorylated. The phosphorylated RTKs then bind to the SRC-homology-2 domains (SH2 domains) of the p85 subunit of PI3K (Okkenhaug and Vanhaesebroeck 2001). The interaction between RTKs and p85 leads to the activation of p110, which then generates phosphatidylinositol 3,4,5-trisphosphate (PIP₃) from phosphatidylinositol 4,5-bisphosphate (PIP₂). PIP₃ serves as a second messenger to recruit protein kinase B (PKB), also known as Akt, to the membrane by binding to the pleckstrin-homology (PH) domain of Akt (Corvera *et. al.* 1998). The level of PIP₃ is tightly regulated by phosphatase and tensin homologue (PTEN), which is a tumor suppressor capable of converting PIP₃ back to PIP₂ (Maehama and Dixon 1998). Once in the membrane, Akt is phosphorylated first at threonine 308 in the kinase domain by a serine/threonine kinase called 3-phosphoinositide-dependent protein kinase-1 (PDK1; Alessi *et. al.* 1997). Full activation of Akt requires the phosphorylation at a second site, serine 473, in the C-terminal hydrophobic tail. The protein responsible for phosphorylating Akt at serine 473 remains controversial, but possible candidates include integrin-linked kinase (ILK; Delcommenne *et. al.* 1998), Akt itself (Toker and Newton 2000), DNA-dependent protein kinase (DNA-PK; Feng *et. al.* 2004), the rictor-mTOR complex (Sarbasov *et. al.* 2005), and an yet to be identified “PDK2” (Alessi *et. al.* 1997, Toker and Newton 2000). Once Akt is fully activated, it acts as a master switch that turns on a spectrum of processes necessary to promote cell survival and growth.

1.2.2 The Functions of Akt

Akt, originally identified as human homologues of the viral oncogene *v-akt*, has three

isoforms: Akt1, Akt2, and Akt3 (Staal *et. al.* 1988, Cheng *et. al.* 1992, Masure *et. al.* 1999, Nakatani *et. al.* 1999). Akt isoforms, encoded by distinct but highly related genes, share more than 80% identity at the amino acid level (Nicholson and Anderson 2002). Akt1 and Akt2 are widely expressed whereas Akt3 is predominantly expressed in the brain, heart, and placenta (Masure *et. al.* 1999, Nakatani *et. al.* 1999). All Akt isoforms are activated by the same mechanism but may exert different functions. It has been shown that *Akt1* knockout mice exhibit increased apoptosis and growth retardation (Cho *et. al.* 2001, Chen *et. al.* 2001). On the other hand, *Akt2* knockout mice were normal in size but displayed a diabetic phenotype (Cho *et. al.* 2001). Studies on *Akt3* knockout mice suggested that Akt3 may be involved in brain development because these mice had reduced brain size but grew normally and exhibited functional glucose metabolism (Tschopp *et. al.* 2005). Mice lacking both *Akt1* and *Akt2* were viable but died shortly after birth. The mice were dwarfs with abnormal skin and bone development (Peng *et. al.* 2003). Although Akt isoforms may exert specific functions, it is suggested that Akt isoforms share many substrates in the cellular survival and growth pathways as all Akt isoforms are able to induce tumor formation, which will be discussed in section 1.2.3.

Akt is involved in many cellular pathways including cell apoptosis, proliferation, growth and angiogenesis (Figure 1). Akt inhibits apoptosis by directly phosphorylating and inactivating the pro-apoptotic factor BAD (BCL2-antagonist of cell death) and a pro-death protease caspase-9 (Batta *et. al.* 1997, Cardone *et. al.* 1998). Akt also indirectly inhibits apoptosis by regulating components of the cell-death system. For example, Akt phosphorylates the Forkhead family of transcription factors, FOXO1/FKHR (forkhead in rhabdomyosarcoma) and FOXO4/AFX (ALL1 fused gene from chromosome X), and prevents their nuclear translocation, thus inhibiting the transcription of FKHR gene targets including several pro-apoptotic proteins such as BIM and FAS ligand (Brunet *et. al.* 1999). Moreover, Akt

activates MDM2, which targets the pro-apoptotic tumor suppressor p53 for degradation (Mayo and Donner 2001, Zhou *et. al.* 2001b). Furthermore, by phosphorylating the I κ B kinase (IKK), which induces the degradation of the NF- κ B inhibitor (I κ B), Akt indirectly activates the nuclear factor kappa B (NF- κ B). NF- κ B is then able to promote the expression of pro-survival genes (Nicholson and Anderson 2002, Romashkova and Makarov 1999, Kane *et. al.* 1999).

Akt exerts a positive effect on cell viability not only by inhibiting apoptosis, but also by enhancing proliferation through the cell-cycle machinery. For example, Akt phosphorylates glycogen synthase kinase-3 β (GSK3 β), thereby blocking the ability of GSK3 β to target cyclin D1 for degradation. The level of cyclin D1 is retained, and cell cycle progression occurs (Diehl *et. al.* 1998). In addition, Akt negatively regulates the expression of p21 and p27, which are cyclin-dependent kinase (CDK) inhibitors (Zhou *et. al.* 2001a, Viglietto *et. al.* 2002, Liang *et. al.* 2002, Shin *et. al.* 2002). Akt can also promote cell growth by up-regulating components involved in protein translation. The mammalian target of rapamycin, mTOR, which is a serine/threonine kinase that serves as a modulator sensor to regulate protein synthesis based on nutrient availability (Vivanco and Sawyer 2002), is phosphorylated by Akt. Upon phosphorylation, mTOR activates the p70 S6 kinase and the S6 ribosomal protein (Nave *et. al.* 1999), leading to protein translation (Dennis *et. al.* 1996). Activated mTOR also inhibits the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which is a translation repressor (Gingras *et. al.* 1999).

The ability of Akt to regulate both apoptosis and cell growth implies that deregulation of Akt may lead to transforming events. In fact, many studies have demonstrated the oncogenic property of Akt (section 1.2.3). It is interesting to note that Akt can also affect angiogenesis by stabilizing the transcription factor hypoxia inducible factor-1 (HIF-1 α) via mTOR, resulting in increased expression of vascular endothelial growth factor (VEGF; Zhong *et. al.* 2000, Zundel *et. al.* 2000, Laughner *et. al.* 2001). As the fast-growing tumors depend heavily on blood vessels to

acquire oxygen, it is possible that deregulated Akt promotes cancer pathogenesis by “improving” the surrounding environment that is more suited for tumor growth.

1.2.3 The Oncogenic Property of Akt

Immunohistochemical staining on tumor tissues revealed that the level of activated or phosphorylated Akt (pAkt) is associated with cancers of the colon (Itoh *et. al.* 2002), lung (Lee *et. al.* 2002), kidney (Horiguchi *et. al.* 2003), prostate (Malik *et. al.* 2002), and the breast ((Perea-Tenorio *et. al.* 2002, Schmitz *et. al.* 2004). Preclinical study using small animals demonstrated that activated Akt1 transformed NIH3T3 cells, allowing malignant NIH3T3 cells to develop tumor in mice (Sun *et. al.* 2001). Mende *et. al.* further created Akt2 and Akt3 mutants by engineering a membrane-targeting myristylation signal of the c-Src kinase to the amino termini of Akt2 and Akt3. The myristylated Akt2 and Akt3, which exhibited kinase activities, were able to induce tumors in animals (Mende *et. al.* 2001).

The role of pAkt in clinical breast cancer is more controversial. It has been shown by immunohistochemistry that pAkt is not associated with patient survival (Panigrahi *et. al.* 2004, Kucab *et. al.* 2005). In other studies, however, high level of pAkt correlated with relapse and distant metastasis (Perea-Tenorio *et. al.* 2002, Schmitz *et. al.* 2004). It was also demonstrated that pAkt was elevated in approximately 40% of breast cancers, most of which are in stage III/IV (Sun *et. al.* 2001). Moreover, *AKT2* was found to be amplified in 3% of breast cancers and was associated with poor prognosis (Bellacosa *et. al.* 1995). Data from both *in vivo* and *in vitro* work support the idea that activated Akt contributes to breast cancer pathogenesis. First of all, activated Akt is able to transform breast epithelial cells into malignant phenotypes (Zhao *et. al.* 2003, Zhang *et. al.* 2003). In nude mice, co-expression of constitutively active Akt 1 and mutant polyomavirus T antigen resulted in mammary tumor formation in nude mice (Hutchinson *et. al.*

2001). In addition to tumorigenesis, Akt is also involved in tumor progression and metastasis. For example, exogenous over-expression of Akt1 enhanced breast cancer cell growth both in monolayer and soft agar assays (Ahmad *et. al.* 1999). Akt was also shown to promote the motility and invasion of breast cancer cells by increasing the osteopontin mRNA and protein levels (Zhang *et. al.* 2003) as well as the metalloproteinase, MMP-9, protein level (Kim *et. al.* 2001). Besides, Akt2 also mediates invasion and metastasis in breast cancer cells by up-regulating $\beta 1$ integrins protein expression (Arboleda *et. al.* 2003). Furthermore, activated Akt promotes resistance to radiotherapy and chemotherapeutic drugs including doxorubicin, trastuzumab, and tamoxifen by suppressing apoptosis (Liang *et. al.* 2003, Clark *et. al.* 2002). Together, these studies suggest an important role of activated or phosphorylated Akt in breast cancer.

1.2.4 Akt as a Molecular Therapeutic Target for Breast Cancer

The importance of pAkt in breast cancer has prompted researchers to develop therapies that block the activity and downstream effects of Akt. There are three approaches 1) targeting Akt itself, 2) blocking Akt upstream signaling, or 3) inhibiting Akt downstream substrates. Despite the effort in developing drugs that inhibit Akt or interfere with Akt:PIP₃ interaction (Lin *et. al.* 2004, Castillo *et. al.* 2004), there are currently no Akt small molecule inhibitors on the market. However, adenoviral-mediated expression of a kinase-dead mutant of Akt, in which both Thr308 and Ser473 were changed to alanine, is able to induce apoptosis selectively in tumor cells and to reduce tumor growth in mice (Jetzt *et. al.* 2003). This study provided proof of principle that inhibiting Akt can suppress cancer progression.

Targeting RTKs has been a popular therapeutic strategy for breast cancer. ERBB2, for example, is over-expressed in about 30% ER(-) aggressive breast carcinoma and is associated

with poor prognosis and increased risk of relapse (Slamon *et. al.* 1987). Trastuzumab (Herceptin) is a humanized monoclonal antibody developed to target ERBB2. By preventing ligand-ERBB2 interaction, this antibody has shown an excellent clinical anti-tumor profile and was approved by the US Food and Drug Administration in 1998 for treating ER(-) ERBB2-overexpressing metastatic breast cancer (Baselga 2001, Slamon *et. al.* 2001). However, ERBB2- overexpressing breast cancer patients whose tumors are PTEN-negative often develop resistance to trastuzumab (Nagata *et. al.* 2004). In addition to ERBB2, the epidermal growth factor receptor (EGFR or ERBB1), is also commonly over-expressed in breast carcinoma (Salomon *et. al.* 1995, Osaki *et. al.* 1992, Klijn *et. al.* 1992, Bucci *et. al.* 1997). The expression of EGFR has been shown to be associated with tumor aggressiveness and poor prognosis in breast cancer patients (section 1.4.4). Therefore, there has been great interest in developing drugs to target EGFR to treat breast cancer. The small molecule inhibitor, gefitinib (also known as Iressa™; AstraZeneca, London, UK), which competes with ATP and inhibits the kinase domain of EGFR, is already approved for the treatment of patients with advanced non-small-cell lung cancer (NSCLC; Cohen *et. al.* 2004). Gefitinib therefore serves as an attractive candidate for breast cancer therapy. Unfortunately, the efficacy of gefitinib in treating metastatic breast tumors was found to be low in phase II studies (Albain *et. al.* 2002, von Minckwitz *et. al.* 2005). To date no clinical trials of gefitinib focusing on ER(-)/EGFR-overexpressing breast tumors have been reported. However, another phase II clinical trial of gefitinib revealed that it was largely ER(+) breast cancer patients who were resistant to tamoxifen and express relatively modest EGFR obtained clinical benefit from gefitinib (Robertson *et. al.* 2003, Gutteridge 2004). Patients with ER(-)/EGFR(+) breast tumors, however, did not respond very well to the drug (Robertson *et. al.* 2003, Gutteridge 2004). In fact, the efficacy of gefitinib in NSCLC seems to be independent of EGFR expression. It was recently discovered that gefitinib is only effective in NSCLC patients who harbor a specific mutation in

EGFR (Lynch *et. al.* 2004). Such a mutation has not been reported in breast tumors. In addition to the ERBB family, IGF-1R is implicated in breast cancer invasion and metastasis (Kucab and Dunn 2003), which led to the development of antibodies or small molecule inhibitors to target IGF-1R (Burtrum *et. al.* 2003, Garcia-Echeverria *et. al.* 2004, Mitsiades *et. al.* 2004). However, the efficacy of drugs targeting IGF-1R still requires further evaluation.

In addition to drugs that target RTKs, small molecules including wortmannin and LY294002 that inhibit PI3K activity were developed. However, these drugs are not suitable for treating cancers as wortmannin is not stable in an aqueous environment while LY294002 is too toxic. Recently, the cyclooxygenase-2 (COX-2) inhibitor, celecoxib, was discovered to inhibit PDK-1. Celecoxib derivatives, termed OSU-03012 and OSU-03013, were then developed to specifically target PDK-1 and not COX-2. These orally available analogs showed improved PDK1 inhibitory ability and were able to induce apoptosis by blocking Akt (Zhu *et. al.* 2004). Our lab previously demonstrated the ability of OSU-03012 and OSU-03013 to induce apoptosis in breast cancer cells (Kucab *et. al.* 2005). We also showed in breast cancer cells that OSU-03012 specifically inhibits PDK-1 without affecting other signaling pathways (Kucab *et. al.* 2005). Our lab felt that OSU-03012 may be a good drug candidate for treating breast tumors, and one of the objectives of this study is to further evaluate the potential of OSU-03012 in breast cancer therapy.

Given the role of Akt2 in glucose metabolism, targeting Akt downstream substrates may be a preferable approach to treat breast cancer. Rapamycin, which is an immuno-suppressant, inhibits mTOR and is expected to have anti-proliferative effect on cancers (Vara *et. al.* 2004). Poor solubility and chemical instability of rapamycin led to the development of analogs with more favorable pharmaceutical characteristics (Carraway 2004). The rapamycin analog CCI-779 has shown good tolerability and response rates in clinical trials (Chan *et. al.* 2005). The ongoing

phase III study will help define the role of this therapy in breast cancer (Morgensztern and McLeod 2005). Two other rapamycin analogs, RAD001 and AP23573, are in the early phase of development (Morgensztern and McLeod 2005).

For the past few years, our lab has been focusing on identifying novel Akt downstream substrates specifically in the context of breast tumors in hope of developing alternative therapies for treating breast cancer. We screened tumor tissue microarrays (TMAs) representing 500 breast cancer patients and found that, among approximately 30 proteins examined, pAkt is co-expressed with a transcription/translation factor called the Y-box binding protein-1 (YB-1). Subsequent *in vitro* kinase assays indicated that Akt forms a complex with YB-1 and phosphorylates it on Ser102, which is located in the cold-shock domain (CSD). Since YB-1 is known to be over-expressed in breast cancer (Bargou *et. al.* 1997, Rubinstein *et. al.* 2002, Janz *et. al.* 2002), our finding was significant as it suggested that PI3K/Akt may regulate YB-1 and that the action of these two proteins may contribute to breast cancer development or progression. Therefore, for the first part of the project we will elucidate whether YB-1 is phosphorylated at Ser102 through PI3K/Akt signaling and how Ser102 contributes to YB-1 functioning.

1.3 The Y-box Binding Protein-1 (YB-1)

The *YB-1* gene is located on chromosome 1p34 and comprises of eight exons (Toh *et. al.* 1998). The mRNA is approximately 1.5 kb long and encodes a protein with 324 amino acids (Didier *et. al.* 1988). YB-1 was first isolated as a transcription factor that bound to the promoter of the major histocompatibility complex class II (MHC Class II). YB-1 was named as it recognized the Y-box in the MHC Class II promoter. The Y-box identified in the MHC Class II promoter is a 14-nucleotide conserved sequence that contains the inverted CCAAT box (Okada *et. al.* 1985), and the inverted CCAAT box is required for YB-1 binding (Didier *et. al.* 1988). At the

same time, another group identified a DNA binding protein that interacted with the EGFR enhancer (Sakura *et. al.* 1988). This protein, referred to DNA binding protein B (DbpB), was later shown to be identical to YB-1. Soon after, YB-1/DbpB was suggested to be the same as the nuclease-sensitive element protein-1 (NSEP-1), which was independently cloned based on its ability to bind to CT-rich elements in the *C-MYC* promoter (Kolluri and Kinniburgh 1991). More recently, YB-1 has also been called the human homolog of p50, which was originally found to be one of the major proteins in messenger ribonucleoprotein particles (mRNPs) in rabbit reticulocytes (Evdokimova *et. al.* 1995).

1.3.1 Gene Family and Expression

YB-1 is a member of the Y box protein family. These proteins were identified in a number of eukaryotic and prokaryotic organisms, including human YB-1, DNA-binding protein A and C (DbpA or C; Tekur *et. al.* 1999); rat EF1A (Ozer *et. al.* 1990); frog FRGY1 and FRGY2 (Tafuri and Wolffe 1990); mouse MUSY1 and MUSY2 (Tafuri *et. al.* 1993, Yan and Tamm 1991); chicken chkYB-1 (Grant and Deeley 1993); rabbit p50 (Evdokimova *et. al.* 1995); and bacterial cspA and cspB (Wolffe 1993). In humans, while YB-1 has been studied extensively, little is known about DbpA and DbpC. Since DbpA is expressed in the heart and muscle whereas DbpC is detected in germ cells (Tekur *et. al.* 1999), perhaps their functions are tissue-specific.

On the other hand, the expression of YB-1 varies among tissues and developmental stages. For example, YB-1 is widely expressed in fetal cerebrum, heart, muscle, liver, bone marrow, and kidney (Spitkovsky *et. al.* 1992). In addition, YB-1 is also produced in cell lines including fibroblasts, T-cells, B-cells, and monocytes (Spitkovsky *et. al.* 1992). However, YB-1 mRNA is not detected or is expressed at very low level in many adult tissues including breast tissues (Spitkovsky *et. al.* 1992), suggesting the expression of YB-1 must be tightly regulated.

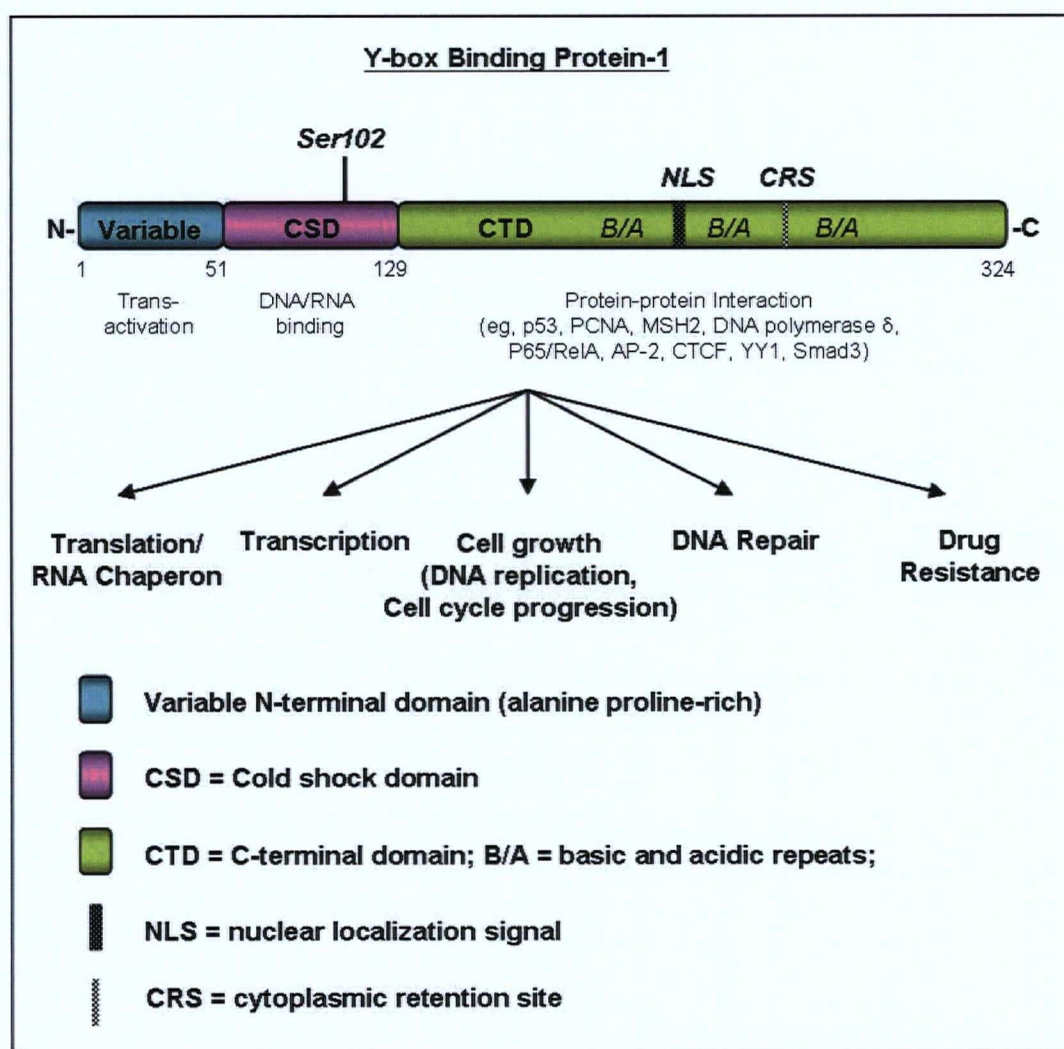
Multiple E-boxes and GF-boxes on the *YB-1* gene promoter have been shown to be bound by c-Myc, which interacts with p73 to regulate YB-1 expression (Uramoto *et. al.* 2002). Another transcription factor, GATA-1, also binds to the 5'-UTR and activates the *YB-1* promoter (Yokoyama *et. al.* 2003). Notwithstanding, mechanisms that control YB-1 expression remain largely unknown.

1.3.2 Protein Structure and Function

YB-1, like all other vertebrate Y-box binding proteins, consists of three domains (Figure 2): a non-conserved variable N-terminal domain, a highly conserved cold-shock domain (CSD), and a C-terminal domain (CTD; Kohno *et. al.* 2003). The N-terminal domain, which is rich in alanine and proline residues, is involved in transactivation (Kohno *et. al.* 2003). The CSD, which contains two conserved RNP motifs (Graumann and Marahuel 1996), is homologous to bacterial cold-shock proteins (Wolffe *et. al.* 1992). In bacteria, cold-shock proteins respond to low-temperature by sustaining the expression of genes necessary for cell growth (Jones and Inouye 1994). The role of eukaryotic YB-1 in cold shock response is less defined, with only one report showing that at 33°C cells with the *YB-1* locus disrupted do not replicate while the wild type cells grow slowly (Matsumoto *et. al.* 2005). It therefore seems that the CSD of eukaryotic YB-1 may not be involved in the cold shock response. In fact, the main function of CSD is to bind to oligonucleotides including RNA, double stranded DNA, and single strand DNA (Bouvet *et. al.* 1995, Didier, *et. al.* 1988, Hasagawa *et. al.* 1991, Kolluri *et. al.* 1992, MacDonald *et. al.* 1995). The CTD of YB-1 contains alternating regions of basic and acidic residues, termed charged zipper or the B/A repeats, and is suggested to mediate protein-protein interactions (Tafari and Wolffe 1992, Swamynathan *et. al.* 1998, Nambiar *et. al.* 1998, Bouvet *et. al.* 1995). Proteins shown to interact with YB-1 include PCNA, MSH2, and DNA polymerase δ (Ise *et. al.*

Figure 2
The Structure and Functions of YB-1

YB-1 contains an N-terminal variable domain which is rich in alanine and proline residues, a cold shock domain in which Ser102 is located, and a C-terminal domain which contains acidic and basic repeats. The nuclear localization signal and the cytoplasmic retention signal are also located within the B/A repeats in the C-terminal domain. YB-1 is multi-functional and is involved in translation, transcription, cell growth, DNA repair, and drug resistance.



1999, Gaudreault *et. al.* 2004). These alternating blocks of basic and acidic residues are also a common feature of proteins that bind to ribonucleoprotein complexes to shuttle between the nucleus and cytoplasm (Ranjan *et. al.* 1993). Noncanonical nuclear localization signals (NLS) and a cytoplasmic retention site (CRS) were also identified in CTD to control YB-1 cellular localization (Bader *et. al.* 2005). The mechanism for YB-1 trafficking remains unclear. It has been shown that the thrombin protease induced YB-1 nuclear translocation (Stenina *et. al.* 2001). However, whether the NLS and the CRS are involved in this process needs further investigation.

1.3.3 The Pleiotropic Functions of YB-1

YB-1 is a multi-functional protein involved in a multitude of biological processes. YB-1 shuttles between the cytoplasm and the nucleus. While in the cytoplasm, YB-1 acts as a translation factor. Once in the nucleus, YB-1 regulates transcription. Moreover, YB-1 is also involved in cell growth, DNA repair, and drug resistance (Figure 2).

1.3.3.1 YB-1 in Translation

YB-1 was identified as a major component of mRNPs and is essential for initiating translation (Evdokimova *et. al.* 1995, 1998). While the CSD binds to the sequence “AACAU”, the CTD interacts with RNA non-specifically (Bouvet *et. al.* 1995). Both the CSD and the CTD of YB-1 are required for incorporating mRNP into mRNA (Matsumoto *et. al.* 1996). Interestingly, whether YB-1 activates or represses translation depends on its concentration in a cell. It was shown in several *in vitro* studies that at low level, YB-1 binds to mRNA as monomers and “opens” up the transcript for translation, either by exposing the mRNA structure to translation factors or by facilitating 5'-UTR scanning. On the other hand, high concentration of YB-1 leads to homo-multimerization on mRNA to form the “beads-on-string” structure, preventing the

access of translation factors to the transcript (Evdokimova *et. al.* 1998, Skabkin *et. al.* 2004). YB-1 was recently shown to interfere with the translation initiation factor, eIF4G, to bind to mRNA, resulting in inhibition of translation (Evdokimova *et. al.* 2001, Nekrasov *et. al.* 2003). Moreover, the “beads-on-string” type of structure also suggests a role of YB-1 in packaging and storing cytoplasmic mRNA (Evdokimova *et. al.* 1995, Skabkin *et. al.* 2004). This hypothesis was further supported by the discovery that YB-1 binds to the cap (m⁷GpppG or GpppG) of mRNA thereby stabilizing the transcript by preventing degradation (Evdokimova *et. al.* 2001, Chen *et. al.* 2000). In addition to translation initiation and mRNA stabilization, YB-1 is also involved in recognizing alternative splice sites and stimulating pre-mRNA splicing (Chansky *et. al.* 2001, Stickeler *et. al.* 2001, Raffetseder *et. al.* 2003). While YB-1 binds mRNA and affects translation, the association between YB-1 and mRNA seems to in turn stabilize YB-1 in the cytoplasm. It has been shown by Sorokin *et. al.* that dissociation from mRNA leads to proteasomal cleavage of YB-1 (Sorokin *et. al.* 2005). Whether the cleaved YB-1 is eventually degraded or translocates into the nucleus, like the thrombin-cleaved YB-1 (section 1.3.2), was not determined in the study.

1.3.3.2 YB-1 in Transcription

As a transcription factor, YB-1 binds to the inverted CCAAT element in the Y-box (Didier *et. al.* 1988, Goldsmith *et. al.* 1993, Wilfred *et. al.* 1997, Norman *et. al.* 2001, Jürchott *et. al.* 2003). YB-1 is also able to recognize Y-box without the consensus inverted CCAAT box (Mertens *et. al.* 1997, Higashi *et. al.* 2003, Lasham *et. al.* 2003), and sequences flanking the Y-box have been suggested to contribute to YB-1:DNA interactions as well (Didier *et. al.* 1988, Norman *et. al.* 2001). YB-1 is implicated in a wide range of cellular processes by functioning as either a transcription activator or repressor.

First of all, YB-1 is involved in immune regulation as it blocks the expression of the

class II major histocompatibility complex (Class II MHC) induced by interferon γ (IFN- γ ; Didier *et. al.* 1988, Ting *et. al.* 1994). The expression of FAS (Apo-1/CD95), which activates T cells and stimulates activated-induced cell death, is also suppressed by YB-1 (Lasham *et. al.* 2000). Considering the repressing role of YB-1 in the immune system, it appears to be a clever choice by pathogenic viruses to use YB-1 to escape from immuno-surveillance and to activate their own transcription. Indeed, YB-1 has been shown to regulate gene expression of many viruses, such as the Rous sarcoma virus d (RSV-d; Kandala and Guntaka 1994)), the human neurotropic papovavirus JC virus (JCV; Kerr *et. al.* 1994, Raj *et. al.* 1996, Safak *et. al.* 1999, 2002), the human T-cell lymphotropic virus type I (HTLV-I; Kashanchi *et. al.* 1994), the human immunodeficiency virus type 1 (HIV-1; Sawaya *et. al.* 1998), and adenovirus (Holm *et. al.* 2002). In the case of JCV, YB-1 was shown to regulate transcription by interacting with the JCV large T antigen (Safak *et. al.* 1999), agnoprotein (Safak *et. al.* 2002), and the NF- κ B/rel family protein, p65/relA (Raj *et. al.* 1996). It is interesting to note that among these viruses, only adenoviral and HIV-1 promoters contain the inverted CCAAT box (Holm *et. al.* 2002), suggesting the existence of other regulatory sequences that can also be recognized by YB-1.

Secondly, YB-1 is involved in cell growth. YB-1 up-regulates the transcription of *CYCLIN A* (Jürchott *et. al.* 2003), *CYCLIN B1* (Jürchott *et. al.* 2003), *TOPOISOMERASE II α* (Shibao *et. al.* 1999), and *DNA POLYMERASE α* (En-Nia *et. al.* 2005), implying that YB-1 enhances cell growth possibly by promoting both cell cycle progression and DNA replication. It was further demonstrated by Swamynathan *et. al.* that cells with targeted disruption of one allele of *YB-1* displayed defects in cell cycle and reduction in cell growth (Swamynathan *et. al.* 2002). A role of YB-1 in DNA replication, on the other hand, is not as clear. Although it has been suggested that YB-1 interacts with the proliferating cell nuclear antigen (PCNA; Ise *et. al.* 1999) and DNA polymerase δ (Gaudreault *et. al.* 2004), whether YB-1 is part of the DNA replication

holoenzyme needs further investigation.

Due to its role in cell growth, YB-1 has been implicated in cancer pathogenesis (section 1.3.4). In addition to inducing the growth-promoting genes described above, YB-1 was shown to control the expression of phosphatase PTP1B (Fukuda and Tonks 2003), matrix metalloproteinase-2 (MMP-2; Mertens *et. al.* 1997, 2002), matrix metalloproteinase-12 (MMP-12; Samuel *et. al.* 2005), collagen α 1(I) (Norman *et. al.* 2001), and collagen α 2(I) (Higashi *et. al.* 2003), all of which are involved in cell adhesion, motility, and thus metastasis. The transcriptional activity of YB-1 on MMP-2 was demonstrated to be dependent on the interaction with two other transcription factors, namely the activating protein-2 (AP-2) and p53 (Mertens *et. al.* 2002). Furthermore, YB-1 promotes the expression of several players in drug resistance, including p-glycoprotein encoded by the multi-drug resistance gene (*MDR1*; Goldsmith *et. al.* 1993, Stein *et. al.* 2001), multi-drug resistance-related protein-1 (MRP 1; Stein *et. al.* 2001), as well as the major vault protein (MVP; Stein *et. al.* 2005). The ability of regulating genes related to metastasis and drug resistance implies that YB-1 may be a marker for tumor aggressiveness and may predict poor response to chemotherapies.

1.3.3.3 YB-1 in DNA Repair

YB-1 is suggested to be part of the cellular DNA repair system. It was shown by Lasham *et. al.* that YB-1 decreased p53 expression and thus p53 activity, suggesting YB-1 as a negative regulator of DNA repair (Lasham *et. al.* 2003). However, this is contradictory to another study demonstrating that YB-1 enhanced the activity of p53 (Okamoto *et. al.* 2000). The exact role of YB-1 in DNA repair requires further investigation, but there have been other data supporting the idea that YB-1 promotes DNA repair. For example, YB-1 was found to form complexes with the proliferating cell nuclear antigen (PCNA; Ise *et. al.* 1999), the MutS

homologue 2 (MSH2; Gaudreault *et. al.* 2004), and DNA polymerase δ (Gaudreault *et. al.* 2004), all of which are involved in mismatch repair. Besides, YB-1 is able to bind to the Ku antigen, which is a protein implicated in base excision repair (Gaudreault *et. al.* 2004). The Werner syndrome (WRN) protein involved in both non-homologous end-joining and long patch base excision repair also interacts with YB-1 (Gaudreault *et. al.* 2004). Furthermore, YB-1 possesses several characteristics important for DNA repair. First of all, YB-1 is able to bind to single stranded DNA (Spitkovsky *et. al.* 1992, Gaudreault *et. al.* 2004), depurinated DNA (Hasegawa *et. al.* 1991), cisplatin-modified DNA (Ise *et. al.* 1999, Gaudreault *et. al.* 2004), as well as mismatched DNA pairs (Gaudreault *et. al.* 2004). Secondly, YB-1 acts as both 3'→5' exonuclease (Izumi *et. al.* 2001) and endonuclease (Gaudreault *et. al.* 2004) that may excise damaged DNA. Although these studies introduced a novel enzymatic function of YB-1, no further studies have been reported. Finally, it was demonstrated that YB-1 induces single-stranded conformation in DNA (MacDonald *et. al.* 1995). On the contrary, it was shown by another group that YB-1 also promotes DNA annealing (Skabkin *et. al.* 2001). The reason for the contradiction is unknown, but it was proposed that the ability of YB-1 to affect both DNA strand separation and renaturation may be associated with DNA repair (Skabkin *et. al.* 2001).

1.3.3.4 YB-1 and Drug Resistance

As described in section 1.3.3.2, YB-1 induces the expression of *mdr1* and *mrp1*, which encode the drug efflux pumps, ABC transporter P-glycoprotein and MRP1, respectively (Goldsmith *et. al.* 1993, Stein *et. al.* 2001). YB-1 also facilitates the expression of MVP, which is another important player of drug resistance (Stein *et. al.* 2005). Over-expression of YB-1 has been shown to cause cells to become resistant to doxorubicin and etoposide (Bargou *et. al.* 1997), whereas knock down of YB-1 using antisense approaches sensitized cells to cisplatin and

mitomycin C (Ohga *et. al.* 1996, 1998). The role of YB-1 in drug resistance is further demonstrated by the report illustrating that mouse embryonic stem cells with targeted disruption of one allele of *YB-1* ($YB-1^{+/-}$) exhibited increased sensitivity to cisplatin and mitomycin C (Shibahara *et. al.* 2004). Moreover, upon exposure to UV radiation and anticancer drug such as doxorubicin and 5-fluorouracil, (Koike *et. al.* 1997, Ohga *et. al.* 1998, Bargou *et. al.* 1997, Stein *et. al.* 2005), YB-1 shuttles immediately into the nucleus followed by up-regulation of the drug resistance genes. This data is consistent with the finding that high level of YB-1 is detected in the nucleus of resistant cells (Ohga *et. al.* 1996). Immunohistochemical analyses performed in clinical studies have demonstrated the association between nuclear YB-1 and p-glycoprotein expression in cancers of the prostate (Gimenez-Bonafe *et. al.* 2004), bone (Oda *et. al.* 1998), ovarian (Kamura *et. al.* 1999), muscle (Oda *et. al.* 2003), and the breast (Bargou *et. al.* 1997, Saji *et. al.* 2003), suggesting YB-1 as a useful marker in predicting the effectiveness of chemotherapies.

1.3.4 The Oncogenic Property of YB-1

While many studies have focused on the role of YB-1 in drug resistance in cancers, there is growing evidence supporting the oncogenic property of YB-1. For example, the expression of YB-1 is controlled by the proto-oncogene *C-MYC* (Uramoto *et. al.* 2002). In turn, YB-1 may down-regulate the tumor suppressor gene *P53* (Lasham *et. al.* 2003) and up-regulate several growth-related genes including *TOPOISOMERASE II α* , *CYCLIN A*, *CYCLIN B1*, and *DNA POLYMERASE α* as described previously (Shibao *et. al.* 1999, Jürchott *et. al.* 2003, En-Nia *et. al.* 2005). Moreover, YB-1 represses the expression of the apoptosis-associated gene *FAS* (Lasham *et. al.* 2000). A recent study also demonstrated a physical interaction between YB-1 and Smad3 and proposed YB-1 as a component of the IFN- γ -Janus kinase (Jak)-STAT pathway to

interfere with transforming growth factor- β (TGF- β) signaling (Higashi *et al.* 2003). All these studies demonstrate a role of YB-1 in cell growth or cell survival processes, and it is not surprising that deregulation of YB-1 is implicated in the pathogenesis of cancer. Indeed, high level of YB-1 has been shown to be associated with many malignancies including colorectal carcinomas (Shibao *et al.* 1999), prostate cancer (Gimenez-Bonafe *et al.* 2004), osteosarcoma (Oda *et al.* 1998), ovarian serous adenocarcinoma (Kamura *et al.* 1999, Yahata *et al.* 2002), lung cancer (Shibahara *et al.* 2001, Gu *et al.* 2001), synovial sarcoma (Oda *et al.* 2003), and breast cancer (Bargou *et al.* 1997, Janz *et al.* 2002, Rubinstein *et al.* 2002, Huang *et al.* 2005, Wu *et al.* 2006).

1.3.5 YB-1 and Breast Cancer

In breast cancer, YB-1 first became recognized because of its ability to induce drug resistance. It has been confirmed that nuclear expression of YB-1 correlates with p-glycoprotein, the product of the *MDR-1* gene, in breast tumor tissues (Bargou *et al.* 1997). As YB-1 is known to promote cell growth and survival, the possibility that YB-1 can promote breast tumorigenesis and progression cannot be ruled out. In fact, it was discovered by several groups that YB-1 is over-expressed in breast carcinoma and that YB-1 is associated with tumor aggressiveness, relapse and poor survival (Bargou *et al.* 1997, Rubinstein *et al.* 2002, Janz *et al.* 2002). It remains unclear whether up-regulation of transcription or gene amplification is the main cause of YB-1 over-expression in breast cancer. Furthermore, it has been shown in a transgenic mouse model that YB-1 induces mammary tumor formation with 100% genetic penetrance. Close examination of the mouse mammary tumors revealed a high content of binucleate cells, most of which are tetraploid. It was speculated that YB-1 may promote breast tumor formation and/or growth by accelerating cell cycle progression and escaping DNA damage check points

(Bergmann *et. al.* 2005). Recently, our lab demonstrated that YB-1, but not the mutant YB-1 in which Ser102 is changed to alanine (YB-1A102), enhances the growth of breast cancer cells both in monolayer and anchorage-independent conditions (Sutherland *et. al.* 2005). Nonetheless, the mechanism by which YB-1 promotes breast tumor cell growth is unknown.

1.4 Epidermal Growth Factor Receptor (EGFR)

As mentioned previously, YB-1 was originally identified as a DNA binding protein that interacts with the regulatory elements of *EGFR* (Sakura *et. al.* 1988). Interestingly, no follow-up studies have been reported. Since EGFR is commonly over-expressed in breast cancer, we questioned whether there is a functional link between YB-1 and EGFR in breast cancer.

1.4.1 Gene Family and Structure

EGFR, a 170 kDa protein, is a receptor tyrosine kinase (RTK). It belongs to the epidermal growth factor (EGF) receptor superfamily that comprises of four closely related members: EGFR (also termed Her-1 or ERBB1), ERBB2/Her-2, ERBB3/Her-3, and ERBB4/Her-4.

The promoter of the *EGFR* gene lacks a characteristic TATA box and CAAT box. It has high GC content and contains multiple transcription initiation sites (Ishii *et. al.* 1985). Both *cis*- and *trans*-acting elements have been suggested to control *EGFR* transcription. For example, multiple Sp-1 sites and an upstream enhancer (between -1429 to -1109) were found in the promoter region (Johnson *et. al.* 1988a, 1988b, Maekawa *et. al.* 1989). A downstream enhancer located in the first intron was also detected (Maekawa *et. al.* 1989). In epidermoid carcinoma cell lines, Sp-1 and the EGFR-specific transcription factor (ETF) were identified to bind and to regulate *EGFR* transcription (Johnson 1988a, Kageyama *et. al.* 1988a, 1988b). In normal

keratinocytes, an EGF-responsive DNA-binding protein-1 (ERDBP-1) was shown to interact with the *EGFR* promoter upon EGF stimulation; however, no functional studies were performed to illustrate the ability of ERDBP-1 to transactivate the *EGFR* promoter (Chen *et. al.* 1993). More recently, AP-1 was shown to bind to several sites in the *EGFR* promoter region, and c-Jun, which is a subunit of AP-1, was able to induce *EGFR* transcription in breast cancer cells and fibroblasts (Johnson *et. al.* 2000, Zenz *et. al.* 2003). Mice lacking c-jun in keratinocytes also exhibited reduced levels of EGFR mRNA and protein (Zenz *et. al.* 2003). In addition to transcription activators, two repressors namely the GC factor (GCF) and EGFR transcriptional repressor (ETR) were capable of repressing *EGFR* transcription by approximately two-fold (Kageyama *et. al.* 1989a, Kageyama 1989b, Hou *et. al.* 1994a).

Finally, environmental signals including EGF, phorbol 12-myristate 13-acetate (PMA), cAMP (Hudson *et. al.* 1990), TGF- β (Hou *et. al.* 1994b), and hypoxia (Nishi *et. al.* 2002) can also induce *EGFR* transcription. In the case of PMA, it was demonstrated in epidermoid and HeLa cells that *EGFR* transcription stimulated by PMA is partly, if not all, through the transcription factor AP-2 (Johnson 1996). In a study performed in osteosarcoma cell lines, the early growth response factor-1 (Egf-1) was shown to play a role in hypoxia induced *EGFR* transcription (Nishi *et. al.* 2002). However, transcription factors that mediate other environmental stimuli to transactivate the *EGFR* promoter have yet to be identified.

1.4.2 Protein Structure and Function

Like other receptors of the family, EGFR has an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular cytoplasmic tyrosine kinase domain (Hynes and Lane 2005). To date, at least ten growth factors were found to bind to the EGF receptors, and each receptor shows different affinity towards the ligand that it interacts with. For EGFR, there

are currently six EGFR-specific ligands, including EGF, transforming growth factor- α (TGF- α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), and epiregulin (Yarden *et. al.* 2001). Upon ligand binding, the extracellular domain of EGFR undergoes conformational change, allowing the formation of homo- or heterodimers with other members of the EGF receptor family. Her-2, for which no specific direct ligand has been identified yet, is the preferred dimerization partner for EGFR (Graus-Porta *et. al.* 1997). Dimerization of the receptors in turn activates the kinase domain resulting in autophosphorylation of the specific tyrosine residues within the cytoplasmic tail. The phosphorylated tyrosine residues then serve as docking sites for adaptor proteins and downstream effectors, which leads to the activation of many cell signaling pathways. EGFR stimulates two major intracellular signaling cascades, the mitogen-activated protein kinase (MAPK) and the PI3K pathways, resulting in several cellular responses such as proliferation, survival, differentiation, and migration (Prenzel *et. al.* 2001, Slichenmyer and Fry 2001, Yarden *et. al.* 2001). After ligand binding and tyrosine kinase activation, the receptor-ligand complex is internalized for degradation or recycling (Yarden *et. al.* 2001).

1.4.3 The Oncogenic Property of EGFR

Signaling through EGFR not only regulates cell proliferation, but also controls a range of processes essential for tumor progression including cell survival, motility, adhesion, angiogenesis, and tumor invasion (Mendelsohn *et. al.* 2000). Direct evidence showing the oncogenic property of EGFR originated from a study demonstrating that over-expression of human EGFR by a retroviral vector induced a transforming phenotype in NIH 3T3 cells (Velu *et. al.* 1987, Di-Fiore *et. al.* 1987). Transgenic mice that over-expressed EGFR also developed tumors in the bladder and the breast (Cheng *et. al.* 2002, Brandt *et. al.* 2000). Enhanced or constitutive EGFR signaling is indeed observed in many tumors, such as lung, colon, ovarian,

and breast cancers (Laskin and Sandler 2004). Elevated EGFR signaling is thought to be caused by increased expression of the receptor and/or the ligands.

1.4.4 EGFR and Breast Cancer

EGFR is commonly over-expressed in breast carcinoma (Salomon *et al.* 1995, Osaki *et al.* 1992, Klijn *et al.* 1992, Bucci *et al.* 1997). The expression of EGFR was found to be inversely correlated with ER (Tsutsui *et al.* 2002, Lebeau *et al.* 2003, Witton *et al.* 2003, Quintela *et al.* 2005, Wu *et al.* 2006). More specifically, Nielson *et al.* found that EGFR was highly expressed in the ER(-) basal-like subtype breast cancer (Nielson *et al.* 2004). This finding corroborated with the fact that over-expression of EGFR is associated with tumor aggressiveness, metastasis, and poor patient prognosis (Klijn *et al.* 1992, Tsutsui 2 *et al.* 002, Nielson 2 *et al.* 004, Wu *et al.* 2006). Unlike other malignancies such as glioblastoma in which the *EGFR* gene is amplified, over-expression of EGFR in breast cancer is generally due to transcriptional up-regulation (Kersting *et al.* 2004). Thus, it is possible that transcription factors that control EGFR expression may be de-regulated and contribute to tumor formation and/or progression. However, the association between EGFR and most of its transcription factors has not been indicated in the context of breast cancer. Recently, our lab showed that EGFR is co-expressed with YB-1 in breast tumor tissues (Wu *et al.* 2006). In addition, it was demonstrated that normal epithelial cells expressing YB-1 cDNA exhibited elevated level of EGFR (Berquin *et al.* 2005). These findings, together with the fact that YB-1 binds to the enhancer of *EGFR*, prompted us to further explore the possibility that YB-1 regulates EGFR expression in breast tumors and that targeting YB-1 may be a therapeutic intervention for treating breast cancers, especially those that are aggressive and over-express EGFR.

1.5 Thesis Hypothesis and Objectives

Hypothesis

YB-1 is a downstream component of the PI3K/Akt pathway and promotes breast cancer cell growth by up-regulating EGFR expression.

Objective 1: To examine whether YB-1 is regulated by PI3K/Akt signaling *ex vivo*

Aim 1: To determine whether YB-1 is phosphorylated via the PI3K/Akt pathway *ex vivo*

Aim 2: To assess whether YB-1 phosphorylation affects its cytoplasmic/nuclear trafficking

Significance: It was previously shown that Akt phosphorylates YB-1 at Ser102 using *in vitro* kinase assays. However, whether Akt phosphorylates YB-1 in the context of a living breast cancer cell has not been demonstrated. In addition, how Ser102 contributes to YB-1 functioning is unknown. It is anticipated that by accomplishing the two aims, the role of YB-1 in PI3K/Akt pathway as well as the importance of Ser102 in YB-1 activity will be determined.

Objective 2: To verify whether YB-1 regulates EGFR expression

Aim 1: To determine whether YB-1 promotes EGFR expression

Aim 2: To examine whether YB-1 interacts with the promoter of *EGFR*

Aim 3: To investigate whether YB-1 activates the promoter of *EGFR*

Significance: It was found that YB-1 and EGFR are co-expressed in primary breast tumors, but evidence demonstrating functional link between the two proteins is lacking. This study will allow us to examine the possibility that YB-1 directly regulates EGFR expression in breast tumors. As well, by mutating Ser102 to alanine, we will be able to assess whether binding to the promoter and thus transcription of *EGFR* by YB-1 is dependent on the phosphorylation of Ser102.

Objective 3: To evaluate whether OSU-03012 interferes with the function of YB-1

Aim 1: To assess whether OSU-03012 inhibits YB-1 nuclear translocation

Aim 2: To examine whether OSU-03012 blocks the ability of YB-1 to bind to the *EGFR* promoter

Significance: If the activity of YB-1 is regulated through the PI3K/Akt pathway, small molecule inhibitors that block Ser102 phosphorylation should potentially inhibit the function of YB-1. By using the PDK1 inhibitor OSU-03012, the idea that Akt, YB-1, and EGFR all participate in the same pathway to promote breast cancer growth can be further verified.

CHAPTER 2

MATERIAL AND METHODS

2.1 Plasmids

YB-1 or YB-1A102 was cloned into the 3x Flag-CMV-10 vector (Sigma, Oakville, ON, Canada) at the *HindIII* and *XbaI* restriction sites within the multiple cloning sites. These plasmids (Flag:YB-1 and Flag:YB-1A102), along with the empty vector (Flag:EV), were transfected into MCF-7 cells to create stable clones as previously described (Sutherland, 2005), or transiently transfected in human embryonic kidney 293 cells (HEK293T) and MDA-MB-468 breast cancer cells for luciferase reporter assays (section 2.11). The pER1 plasmid was obtained from Dr. Alfred Johnson of the National Cancer Institute, MD, U.S. The pER1 construct was originally created by cloning approximately the first 1kb of the EGFR promoter (-1109 to -16) into the *HindIII* restriction site of pGL3-Basic vector containing the firefly luciferase reporter gene (Promega, Madison, WI, USA). The pRL-TK plasmid (Promega, Madison, WI, USA) contains the *Renilla* luciferase gene, and was used as an internal control to measure transfection efficiency in the luciferase reporter assays.

2.2 Cell Culture

MCF-7 (ATCC®# HTB-22; Manassas, VA) and MDA-MB-453 (ATCC®# HTB-131; Manassas, VA, USA) breast cancer cells were cultured in RPMI-1640 without phenol red (Invitrogen, Burlington, ON, Canada), supplemented with 5% fetal bovine serum (FBS) and 100 units/ml of penicillin and streptomycin (Invitrogen, Burlington, ON, Canada). MDA-MB-468 (ATCC®# HTB-132; Manassas, VA, USA) breast cancer cells and HEK293T (ATCC®#

CRL-1573, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Burlington, ON, Canada) containing 10% FBS and 100 units/ml of penicillin and streptomycin. MCF-7 stably transfected with Flag:EV, Flag:YB-1, or Flag:YB-1A102 constructs were maintained in RPMI-1640 without phenol red, containing 5% FBS, 100 units/ml of penicillin and streptomycin, and 400µg/ml G418 (Invitrogen, Burlington, ON, Canada). The expressions of Flag:YB-1 and Flag:YB-1A102 in the stable clones were constantly checked by western blotting (section 2.7). The levels of the transgene products and the endogenous YB-1 can be easily distinguished as the 3xFlag increased the recombinant protein from ~50 to ~55 kDa. All cells were maintained at 37°C with 5% CO₂. Growth medium was changed every three days. At 90% confluency, cells were trypsinized in 0.25% trypsin with EDTA 4Na (Invitrogen, Burlington, ON, Canada), and one-tenth of the cells were subcultured.

2.3 Growth Factor or Drug Treatment

After trypsinizing and re-suspending in growth medium, cells were diluted 10x in trypan blue (Invitrogen, Burlington, ON, Canada) and counted using a hemocytometer. In order to maintain the same confluency between cell lines in experiments, cells were seeded based on the size of the cells. In general MCF-7 and HEK293T cells, which are larger, were plated at lower cell number than MDA-MB-453 or MDA-MB-468.

2.3.1 Activation of PI3K/Akt pathway by IGF-1 in MCF-7 Cells

To activate the PI3K/Akt pathway, 8×10^6 MCF-7 cells (80% confluent) were plated in 150mm² dishes and serum-starved overnight. The next day the cells were treated with insulin-like growth factor-1 (IGF-1; GroPep, Thebarton, SA, Australia) in serum-free medium without antibiotics for 1 hour and harvested by scraping. To block the activation of the PI3K/Akt

pathway by IGF-1, cells were treated with 30 μ M Ly294002 (Sigma, Oakville, ON, Canada) in serum-free medium for 30 minutes before adding IGF-1.

2.3.2 Inhibition of PI3K/Akt pathway by OSU03012 in MDA-MB-453 and MDA-MB-468 Cells

To block the PI3K/Akt signaling in MDA-MB-453 and MDA-MB-468, 1×10^7 cells were plated in 150cm² dishes. Since it takes longer for MDA-MB-453 cells to adhere to the dish, the cells were allowed to settle for 2 days after seeding. The cells were then treated with 10 μ M OSU-03012, which is dissolved in dimethyl sulphoxide (DMSO), in serum-containing medium for 6 hours and harvested by scraping. OSU-03012 was obtained from Dr. Ching-Shih Chen from the Ohio State University, Ohio, USA.

2.4 Cell Lysis and Protein Extraction

2.4.1 Whole Cell Lysis

Harvested cells were washed with ice-cold PBS and pelleted by centrifuging at 5,000 rpm for 5 minutes. The pellets were resuspended in five-packed-cell volumes of modified Radio-Immunoprecipitation Assay (RIPA) buffer (Appendix) supplemented with protease inhibitor cocktail (PIC; Appendix). To extract EGFR from MCF-7 cells, egg lysis buffer (ELB; Appendix) was used instead. The cells were incubated in the lysis buffer for 30 minutes on ice with vortexing every 10 minutes. The cells were then passed through a 22-gauge needle 10 times to release the cellular contents, and the lysates were purified by centrifuging at 13,000 rpm for 10 minutes at 4°C. The protein-containing supernatants were saved and quantified using Bradford assay (section 2.4.3).

2.4.2 Cytoplasmic and Nuclear Fractionation

Harvested cells were rinsed with ice-cold PBS and pelleted by centrifuging at 5,000 rpm for 5 minutes. Cell pellets were resuspended in five-packed-cell volumes of Buffer A (Appendix) supplemented with PIC (Appendix) and incubated for 8 minutes on ice. The cells were then passed 10 times through a 21-gauge needle. The cytoplasmic fraction was separated from the nuclei by centrifuging at 10,000 rpm for 2 minutes at 4°C. The supernatant that contains the cytoplasmic proteins were transferred to a fresh tube, and the nuclei were washed in five-packed-cell volumes of the Borgatti Buffer B (Appendix) supplemented with PIC (Appendix). The nuclei were again pelleted by centrifuging at 10,000 rpm for 2 minutes at 4°C and the supernatant was removed. The nuclei were then resuspended in one-packed-cell volume of the Buffer C (Appendix) supplemented with PIC (Appendix) and incubated for 30 minutes on ice with vortexing every 10 minutes. The nuclear membrane was ruptured by passing through a 26-gauge needle 10 times, or by sonication (amplitude= 40 watts; cycle= 2-3 seconds; 5 cycles) if the total sample volume is less than 100 μ l. The nuclear proteins were then purified by centrifuging at 13,000 rpm for 20 minutes at 4°C. The supernatant, which is the nuclear protein extract, was transferred to a new tube. Both the cytoplasmic and nuclear extracts were quantified by Bradford assay, which is describe in the subsequent section.

2.4.3 Protein Quantification

Proteins were quantified by Bradford assay. To construct standard curves, increasing amounts of bovine serum albumin (1 μ g, 2 μ g, 5 μ g, 10 μ g, and 20 μ g) were incubated in 1 ml of the Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA, USA) for 15 minutes at room temperature for color development. The absorbance of each standard was measured at 595 nm using the GeneQuant Pro spectrophotometer (Biochrom, Cambridge, UK). A graph of

absorbance versus protein concentration was plotted, and the linear regression and the equation of the standard curve were determined. To quantify protein samples, 1 μ l of protein extract was incubated in 1 ml of the dye reagent for 15 minutes at room temperature. The absorbance at 595nm was measured and used to calculate the protein concentration from the equation of the standard curve.

2.5 Immunoprecipitation

To immunoprecipitate YB-1, 500 μ g of whole cell protein extract (section 2.5.1) was diluted in RIPA buffer (Appendix) to give a final volume of 500 μ l. The protein extract was pre-cleared with 50 μ l of Preciphen goat anti-chicken IgY-agarose beads (Aves Labs, Tigard, Oregon, USA) for 2 hours at 4°C with rotation. The Preciphen beads were equilibrated in RIPA buffer beforehand such that the ratio of beads to RIPA buffer is 1 to 3. After 2 hours of pre-clearing, the beads were removed by centrifuging at 6,000 rpm for 5 minutes at 4°C, and the pre-cleared protein extract was incubated with 2 μ g of chicken YB-1_{C-term} antibody or pre-immune chicken IgY as a negative control (Aves Labs, Tigard, Oregon, USA) overnight at 4°C with rotation. The YB-1_{C-Term} antibody, which recognizes the C-terminus of YB-1, was obtained from Dr. Isabelle Berquin of the Wake Forest University, NC, USA. The antibody:protein complexes were incubated with 60 μ l of Preciphen for 3 hours at 4°C with rotation. The beads were then washed in Aves wash buffer A, Aves wash buffer B, and Aves wash buffer C (Appendix), two times each. To elute proteins, the beads were resuspended in 50 μ l of RIPA buffer and 10 μ l of 6x sample loading buffer (Appendix), vortexed briefly, and boiled for 5 minutes. By centrifuging at 6,000 rpm for 5 minutes at 4°C, the supernatant containing the purified proteins was saved and analyzed by western blotting (section 2.6).

2.6 Western Blotting

2.6.1 Electrophoresis

The whole cell, cytoplasmic, or nuclear extract (10-50 μ g) was mixed with 5x sample loading buffer (Appendix) and boiled for 5 minutes. The denatured proteins and 5 μ l of pre-stained broad range standards (Bio-Rad, Hercules, CA, USA) were loaded onto a SDS-polyacrylamide gel composed of a top 3% stacking gel and a bottom 12% or 6% separating gel (Appendix). For immunoprecipitation experiments, one-third of the total immunoprecipitated samples was loaded. The proteins were first compressed in the stacking gel at 82 Volts and then separated at 170 Volts for 1.5 hours. During electrophoresis, the whole system is submerged in running buffer (Bio-Rad, Hercules, CA, USA).

2.6.2 Transfer of Separated Proteins to Nitrocellulose Membrane

The gel containing the separated proteins and a 0.45 μ m pore-size nitrocellulose membrane were first soaked in transfer buffer (Appendix) for 20 minutes. The proteins were transferred to the membrane in chilled transfer buffer for 1 hour at 110 Volts using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membrane or the blot was then stained with Ponceau-S (0.1% in 5% acetic acid; Sigma, Oakville, ON, Canada) for 1 minute and rinsed with distilled water to ensure complete protein separation and proper transfer.

2.6.3 Primary Antibody Incubation and Detection

To minimize non-specific binding, the blot was blocked with 5% non-fat milk in PBS with 0.1% Tween-20 (Fischer Scientific, Ottawa, ON, Canada) for 1 hour at room temperature. The blot was then incubated with primary antibody overnight on a rocking platform at 4°C.

Antibodies against the following proteins were used in western blotting: actin (Cell Signaling, Danvers, MA, USA), total Akt (Cell Signaling, Danvers, MA, USA), phospho-Akt₄₇₃ (Cell Signaling, Danvers, MA, USA), creb (Cell Signaling, Danvers, MA, USA), EGFR (Stressgen, Victoria, BC, Canada), total S6 ribosomal protein (Cell Signaling, Danvers, MA, USA), phospho-S6 ribosomal protein (Cell Signaling, Danvers, MA, USA), and vinculin (Upstate, Charlottesville, VA, USA). The YB-1_{N-Term} antibody, which recognizes the N-terminus of YB-1, was obtained from Dr. Colleen Nelson of the Prostate Centre, Jack Bell Research Centre, BC, Canada. All the antibodies were diluted 1:1000 in 5% BSA in PBS with 0.1% Tween-20. To detect EGFR in the MCF-7 cells on western blots, a dilution of 1:275 of EGFR antibody was used. To detect protein-antibody complexes, the blot was incubated with an anti-mouse or anti rabbit IgG horseradish peroxide-linked antibody (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in 5% non-fat milk in PBS with 0.1% Tween-20 for 1 hour on a rocking platform at room temperature. The blot was then washed three times, 5 minutes each, in PBS with 0.1% Tween-20 and subjected to the enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and film exposure (Kodak, Burnaby, BC, Canada).

2.7 Immunofluorescence

MCF-7 cells expressing either Flag:EV, Flag:YB-1, or Flag:YB-1A102 were plated on coverslips at 50-60% confluency in RPMI-1640 without phenol-red containing 5% FBS and allowed to attach overnight. After 2 days, cells were fixed with 2% paraformaldehyde in PBS for 20 minutes, washed with PBS, and permeabilized with 0.1% Triton X-100 (Sigma, Oakville, ON, Canada) in PBS for 10 minutes at room temperature. The anti-Flag M2 monoclonal antibody (Sigma, Oakville, ON, Canada) was used at a concentration of 20 µg/ml and visualized with Texas red fluorescence-conjugated anti-mouse secondary antibody (1:500 dilution). The nuclei

were stained with a DAPI-containing mounting medium, Vectashield (Vector Laboratories, Burlingame, CA, USA). Cellular images were captured on an Axioplan 2 Zeiss fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) at 400x magnification.

2.8 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from log-growing MCF-7 (4×10^5 cells in 35 mm dish) expressing either Flag:EV, Flag:YB-1, or the Flag:YB-1A102 using the RNeasy mini kit (Qiagen, Mississauga, ON, Canada). The isolated RNA was quantified using the GeneQuant Pro spectrophotometer (Biochrom, Cambridge, UK). The purity of the sample was determined by the ratio of absorbance (260 nm / 280 nm), of which all samples must be greater than 1.5. Isolated RNA (1 μ g) was reverse-transcribed using the SuperscriptTMIII First-Strand Synthesis System (Invitrogen, Burlington, ON, Canada). The cDNA (1 μ l) was then amplified using EGFR specific primers and probes (Applied Biosystem, Foster City, CA, USA) in TaqMan[®] Universal PCR Master Mix (Applied Biosystem, Foster City, CA, USA). Raw fluorescence data was processed to produce threshold cycle (C_T) by the ABI PRISM[®] 7000 Sequence Detection System. The mRNA of the house keeping gene, TATA box binding protein (TBP; Applied Biosystem, Foster City, CA, USA), was included as loading control to assess differences in sample input. Each sample was analyzed in replicates of four on two separate occasions.

2.9 Chromatin Immunoprecipitation (ChIP)

2.9.1 ChIP in MCF-7 Expressing Flag:EV, Flag:YB-1, or Flag:YB-1A102

ChIP assays in MCF-7 expressing Flag:EV, Flag:YB-1, or Flag:YB-1A102 were performed using the ChIP-ITTM kit (Active Motif, Carlsbad, CA, USA). The protocol can be downloaded from the Active Motif website (www.activemotif.com). Buffers and reagents were

provided by the ChIP-IT kit unless otherwise indicated. In brief, 8×10^6 MCF-7 expressing Flag:EV, Flag:YB-1 or Flag:YB-1A102 cells were seeded in 150 mm plates in normal medium and fixed for 10 minutes with 1% formaldehyde the next day. After quenching the reaction with glycine for 5 minutes, cells were lysed in Lysis Buffer supplemented with protease inhibitor cocktail (PIC) with 10 strokes using a dounce homogenizer. The nuclei was isolated and sonicated in Shearing Buffer containing PIC using a Cole Parmer Ultrasonic Processor (Cole Parmer Canada Inc., Anjou, QU, Canada) at 25% power with 10 pulses of 20 seconds each and a 30 second rest on ice between each pulse to give chromatin fragments between 200 bp and 1kb. The nuclear extract was then pre-cleared with salmon sperm DNA/protein G agarose and incubated with 3 μ g of anti-Flag M2 monoclonal antibody (Sigma, Oakville, ON, Canada) overnight at 4°C. The extract incubated with 3 μ g mouse IgG was used as a negative control. Salmon sperm DNA/protein G agarose was added to the DNA:protein:antibody complexes and incubated for 1.5 hours at 4°C. The beads were washed, and the DNA:protein:antibody complexes were eluted from the beads. In order to reduce non-specific binding, the washing steps were modified from the ChIP-IT protocol so that the beads were washed twice with ChIP IP Buffer containing PIC, 5 times with Wash Buffer 1, twice with Wash Buffer 2, and 3 times with Wash Buffer 3. To reverse crosslink and to remove RNA, the immunoprecipitated material was incubated in 200 mM NaCl and 10 μ g RNase A overnight at 65°C. The proteins were then removed by proteinase K treatment, and DNA was purified using the DNA purification mini-columns provided with the kit. Eluted DNA (5 μ l) was amplified by polymerase chain reaction (PCR) and analyzed on an agarose gel (section 2.9.3).

2.9.2 ChIP in MDA-MB-468

The protocol for immunoprecipitating endogenous YB-1:chromatin complexes in

MDA-MB-468 cells was originally adapted from the Upstate Cell Signaling Solutions Chromatin Immunoprecipitation Assay Kit manual. The protocol was modified so that the conditions were optimized for the use of chicken anti-YB-1 antibody and the Preciphen beads (Aves Labs, Tigard, Oregon, USA). MDA-MB-468 cells (9×10^6 cells) were plated in 150 mm dishes in regular growth medium. After OSU-03012 treatment, the cells were fixed with 1% formaldehyde in growth medium for 10 minutes, washed and harvested in cold PBS supplemented with protease inhibitors phenylmethylsulfonyl fluoride (PMSF; Sigma, Oakville, ON, Canada), aprotinin (Sigma, Oakville, ON, Canada), and pepstatin (Sigma, Oakville, ON, Canada). The cell pellet was resuspended in 300 μ l of SDS lysis buffer (Appendix) with protease inhibitors and sonicated as described previously (section 2.9.1). The sonicated lysate was then diluted 5-fold in ChIP dilution buffer (Appendix) and pre-cleared with 100 μ l of Preciphen beads. One-tenth of the diluted lysate was saved as an input control. The Preciphen beads were equilibrated in SDS lysis buffer beforehand such that the ratio of beads to SDS lysis buffer was 1 to 3. After pre-clearing for 2 hours, the lysate was incubated with chicken anti-YB-1 or pre-immune chicken IgY as a negative control overnight with rotation at 4°C, followed by incubation with 100 μ l Preciphen for 2 hours with rotation at 4°C. Immunoprecipitates were then washed once with 1 ml of low salt buffer (Appendix), once with 1 ml of high salt buffer (Appendix), once with 1 ml LiCl buffer (Appendix), and finally twice with 1 ml of TE buffer (Appendix). Protein:DNA complexes were eluted twice by incubating with 250 μ l of elution buffer (Appendix) for 15 minutes with rotation at room temperature. Eluted samples and input control were then treated with 5 M NaCl at 65°C overnight followed by proteinase K (10 μ g; Invitrogen, Burlington, ON, Canada) treatment at 42°C for 1 hour. DNA was purified by phenol/chloroform extraction and alcohol precipitation. The DNA pellet was resuspended in 40 μ l distilled water. PCR was then performed, and products were analyzed on an agarose gel (section 2.9.3).

2.9.3 Polymerase Chain Reaction (PCR)

DNA obtained from ChIP experiments were amplified with 1 unit/ μ l of Platinum Taq polymerase (Invitrogen, Burlington, ON, Canada) by PCR with the following primers:

EGFR1b	5'-TCGCCGCCAACGCCACAAC-3' (forward)
	5'-ACACGCCCTTACCTTTCTTTTCCTCCAG-3' (reverse)
EGFR2a	5'-CCGCGACTTTCCCTCGCATTCT-3' (forward)
	5'-CCTTCCCCCTTTCCCTTCTTTTGTTTTAC-3' (reverse)
EGFR2b	5'-TCCCATTGCTTTCTCTAGTTTGTTTTTC-3' (forward)
	5'-GTCCACCCCATCCCCACTGTTCTTCTC-3' (reverse)
EGFR3	5'-TTCAGCAAACCCATTCTTCT-3' (forward)
	5'-GCTTCCTGCACACCTGGGCTGAG-3' (reverse)
GAPDH	5'-TACTAGCGGTTTTACGGGCG-3' (forward)
	5'-TCGAACAGGAGGAGCAGCGAGCGA-3' (reverse)

The PCR program was set with an initial melting step at 94°C for 3 minutes followed by 35-40 cycles of 94°C for 20 seconds, 59°C for 30 seconds, and 72°C for 30 seconds. The PCR products and 100 bp DNA markers (New England Biolabs, Ipswich, MA, USA) were diluted in 6x DNA loading buffer (Appendix) and then analyzed on a 2% agarose gel by electrophoresis in Tris-Acetate-EDTA buffer (TAE buffer; Appendix). The DNA bands were stained with ethidium bromide and viewed with the Chemi Genius² Bio Imaging Systems (Perkin Elmer, Woodbridge, ON, Canada). The intensities of the DNA bands were quantified using the SynGene Gene Tools and Gene Snap softwares (Perkin Elmer, Woodbridge, ON, Canada).

2.10 Transfection

2.10.1 Transfection in HEK293T Cells

The HEK293T cells were counted by trypan blue exclusion (section 2.3) and seeded in 6-well plates (4×10^5 cells per well). The following day, the cells were transfected in triplicate wells using Lipofectamine™ (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions. In brief, 0.5 µg of pER1, 0.1 µg of pRL-TK, and 0.1-1.0 µg of the expression vector (Flag:EV, Flag:YB-1, or Flag:YB-1A102) were diluted in 50 µl of serum-free DMEM without antibiotics. The co-transfected pRL-TK reporter gene was used as an internal control. In a separate tube, 5 µl of Lipofectamine™ was added in 45 µl serum free medium without antibiotics. The diluted DNA and Lipofectamine™ were then mixed together. The cells were rinsed with warm phosphate buffered saline (PBS; Appendix) and 400 µl of serum free medium without antibiotics was added to each well. The DNA:Lipofectamine complexes were then added drop-wise to the cells. After 5-hour incubation, the medium containing the DNA:Lipofectamine complexes was removed and replaced with regular growth medium without antibiotics. The cells were incubated for another 18-19 hours, and luciferase reporter assay was then performed (section 2.11).

2.10.2 Transfection in MDA-MB-468 Cells

MDA-MB-468 cells were seeded in 6-well plates (5×10^5 per well) and transfected in triplicate wells using Lipofectamine™ 2000 (Invitrogen, Burlington, ON, Canada). DNA (0.5 µg of pER1, 0.1 µg of pRL-TK, and 1.0 µg of either Flag:EV, Flag:YB-1, or Flag:YB-1A102) was diluted in 250 µl serum-free Opti-Mem (Invitrogen, Burlington, ON, Canada). In another tube, 10 µl of Lipofectamine™ 2000 was added to 240 µl of serum-free Opti-Mem and incubated at room temperature for 5 minutes. The diluted DNA and Lipofectamine™ 2000 were mixed

together and incubated at room temperature for 20 minutes. The cells were rinsed with warm PBS, and 2 ml of regular growth medium without antibiotics was added to each well. The DNA:Lipofectamine™ 2000 complexes were then added drop-wise to the cells. The cells were transfected for 5 hours, and the medium containing the transfection complexes was replaced with regular growth medium without antibiotics. The cells were incubated for 18-19 hours, followed by luciferase reporter assay (section 2.11).

2.11 Luciferase Reporter Assay

The luciferase reporter assay was performed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA), and the protocol can be downloaded from the website (www.promega.com). All buffers and reagents were supplied with the kit. In brief, the transfected cells (section 2.4) were washed with cold PBS and incubated with Passive Lysis Buffer (PLB) for 15 minutes. The Luciferase Assay Reagent II (LARII) was then mixed with each lysate, and the firefly luciferase activity was measured using a Lumat LB 9507 luminometer (Perkin Elmer, Fremont, CA, USA). Quenching of firefly luciferase luminescence and simultaneous activation of *Renilla* luciferase was performed by adding the Stop and Glow® Reagent to each sample. The *Renilla* activity was again measured by the same luminometer. The luminometer was programmed to perform a 2-second pre-measurement delay, followed by a 20-second measurement period for each reporter assay. To account for the differences in transfection efficiencies among samples, the activity of the firefly luciferase was normalized to that of the *Renilla* luciferase.

CHAPTER 3

RESULTS

3.1 YB-1 is a Downstream Component of the PI3K/Akt Cascade

3.1.1 YB-1 is Phosphorylated at Ser102 via PI3K/Akt Signaling *ex vivo*

It was previously found that Akt forms a complex with YB-1 and phosphorylates YB-1 specifically at Ser102 in the cold shock domain (CSD; Sutherland *et. al.* 2005) by *in vitro* kinase assays. Thus, we would like to examine phosphorylation of YB-1 by Akt in the context of breast cancer cells *ex vivo*. MCF-7 breast cancer cells, which have low level of phosphorylated or activated Akt (pAkt; Figure 3, lane 3), provided a suitable system in which the PI3K/Akt pathway can be stimulated by growth factors such as the insulin-like growth factor-1 (IGF-1). To activate Akt, MCF-7 cells were serum-starved overnight and treated with IGF-1 for 1 hour. To measure YB-1 phosphorylation at Ser102, the most direct approach is to use a phospho-Ser102-YB-1 specific antibody. Unfortunately, we do not have a suitable antibody for this purpose. In fact, we have attempted to make a phospho-Ser102-YB-1 antibody by two different companies, but the antibodies generated were proven to be non-specific. Alternatively, total YB-1 was immunoprecipitated and phospho-Ser-YB-1 (pSer-YB-1) was detected using a phospho-serine specific antibody by western blotting. We were able to detect a single band, which was considered to represent pSer-YB-1. The molecular weight of pSer-YB-1 appeared to be higher than YB-1 (58 kDa and 50kDa, respectively; Figure 4A, panel 2) in a SDS-PAGE gel. As shown in Figure 4A, basal level of pSer-YB-1 was observed in serum starved cells (Figure 4A, lane 1). Upon treatment of IGF-1, the PI3K/Akt pathway was activated as indicated by the increased level of pAkt (Figure 4A, panel 3). Activation of the pathway also induced serine

Figure 3
Characterization of the Cell Lines Used in the Study

The protein levels of YB-1, phospho-Akt, total Akt, phospho-S6 ribosomal protein, total S6 ribosomal protein, and EGFR were examined by western blotting in cell lines used in the study. Vinculin was used as a loading control. The cell lines used include the human embryonic kidney HEK293T cells as well as two breast cancer cell lines: MCF-7 and MDA-MB-468. The immortalized normal epithelial breast cell line, 184hTERT, was included as a reference.

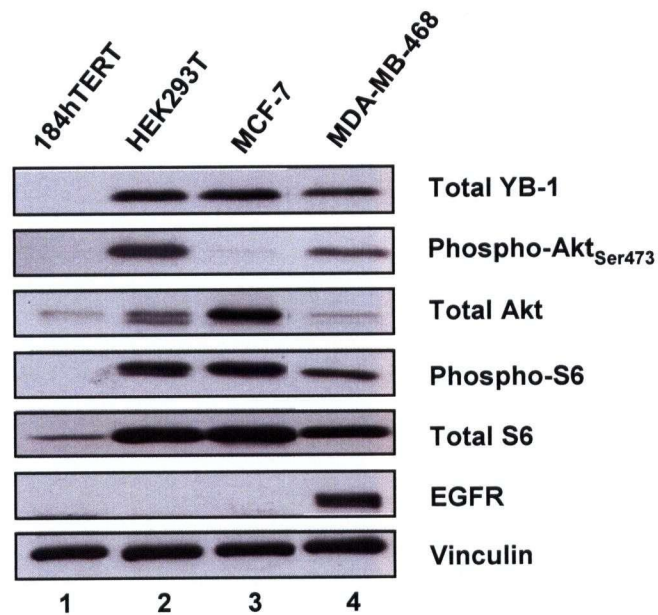
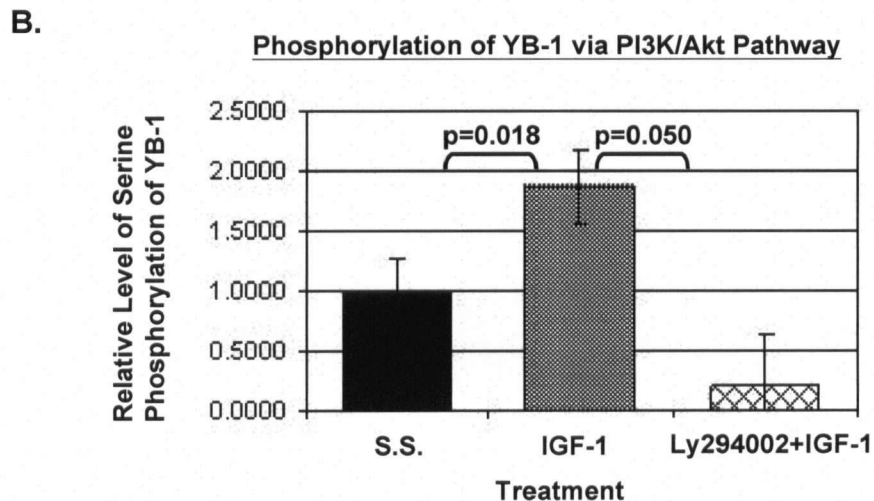
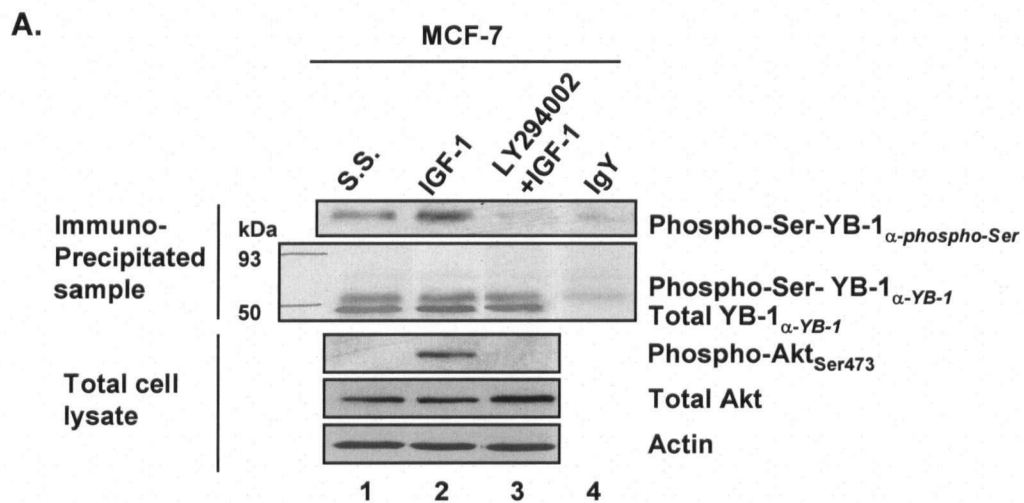


Figure 4**YB-1 was Serine Phosphorylated via the PI3K/Akt Signaling Cascade**

(A) YB-1 was immunoprecipitated from MCF-7 cells that were serum starved (S.S.), IGF-1 stimulated (IGF-1) for 1 hour, or pre-treated with Ly294002 for 30 minutes before IGF-1 stimulation (Ly294002+IGF-1). YB-1 phosphorylation at Ser102 was then detected with a phospho-serine specific antibody on a western blot. Actin was used as a loading control. (B) The experiment was repeated three times. The band intensities of phospho-Ser-YB-1, total-YB-1, and IgY were measured using densitometry, which were then used to calculate the amount of induction or suppression of YB-1 phosphorylation by IGF or Ly294002 with the formula: (phospho-Ser-YB-1 – IgY)/total-YB-1. The basal level of phospho-Ser-YB-1 in serum starved cells was set at “1”. A student t-test was performed to calculate the p-value.



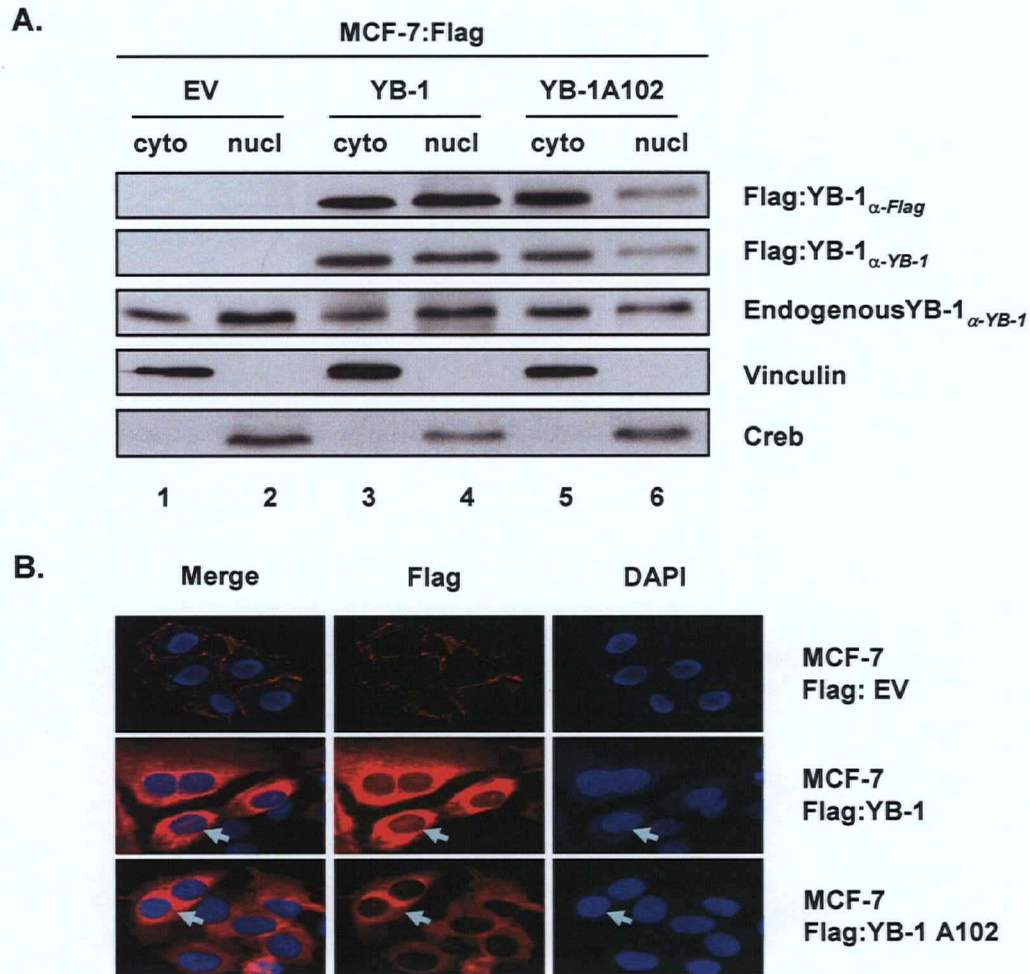
phosphorylation of YB-1 (Figure 4A, lane 2). By measuring the intensities of the bands using densitometry, it was found that IGF-1 stimulation caused almost 1.9 fold increase in YB-1 serine phosphorylation ($p=0.018$; Figure 4B). Ly294002, on the other hand, blocked PI3K/Akt signaling as well as YB-1 serine phosphorylation (Figure 4A, lane 3). It was interesting to note that the level of pSer-YB-1 in Ly294002 treated cells was even lower than that in the untreated cells (Figure 4A, lane 3). Indeed, Ly294002 suppressed YB-1 serine phosphorylation approximately 5 fold ($p=0.05$; Figure 4B), suggesting the basal serine phosphorylation of YB-1 was partly, if not all, induced by the low level of PI3K/Akt signaling in MCF-7 cells. Together these results indicated that the Ser102 residue of YB-1 was phosphorylated through the PI3K/Akt pathway *ex vivo*.

3.1.2 Disruption of Ser102 Suppresses YB-1 Nuclear Translocation

Akt is known to directly or indirectly affect the cytoplasmic-nuclear shuttling of transcription factors by phosphorylation. It has been suggested that the CSD, in which Ser102 is located, cooperates with the C-terminal domain to control the cellular localization of YB-1 (Jürchott *et.al.* 2003). Therefore, we questioned whether Ser102, the site of Akt phosphorylation, plays a role in YB-1 cellular trafficking. To investigate this possibility, the cytoplasmic and nuclear fractions of log-phase proliferating MCF-7 cells expressing either Flag:YB-1 or the mutant YB-1, in which Ser102 is changed to alanine (Flag:YB-1A102), were first analyzed on a western blot. The recombinant proteins were detected using either Flag (Figure 5A, first panel) or YB-1 antibody (Figure 5A, second panel). Flag:YB-1 was detected both in the cytoplasm and the nucleus, (Figure 5A, lanes 3 and 4), whereas Flag:YB-1A102 was retained in the cytoplasm (Figure 5A, lanes 5 and 6). The densities of Flag:YB-1 and Flag:YB-1A102 bands were quantified using densitometry to determine the relative amounts of the recombinant proteins in

Figure 5¹**Loss of Ser102 Suppressed Nuclear Translocation of YB-1**

(A) Cytoplasmic and nuclear proteins of MCF-7 cells were fractionated and examined by western blotting. Cytoplasmic and nuclear Flag:YB-1 and Flag:YB-1A102 were detected using either the Flag or the YB-1 antibodies. The levels of endogenous YB-1 in the cytoplasm and nucleus were also monitored. Vinculin and creb were used as loading controls for the cytoplasmic and nuclear fractions, respectively. (B) The cellular localization of Flag:YB-1 and Flag:YB-1A102 (red signal) was investigated using immunofluorescence. DAPI (blue signal) was used to detect the nucleus. This was performed in collaboration with Min Chen (PhD candidate, Dr. Catherine Pallen's Lab) and presented here with permission.



¹Published in Sutherland *et. al. Oncogene*. 2005.

the cytoplasmic and nuclear fractions. The nuclear/cytoplasmic ratios for Flag:YB-1 and Flag:YB-1A102 were 1.23 and 0.41, respectively, when proteins were detected by YB-1 antibody. Similarly, when measured by Flag antibody, the nuclear/cytoplasmic ratios for Flag:YB-1 and Flag:YB-1A102 were 0.99 and 0.63, respectively. It was noted that the level of endogenous YB-1 in the nucleus of cells expressing Flag:YB-1A102 was lower than that in the nucleus of cells expressing either Flag:EV or Flag:YB-1, implying that the Flag:YB-1A102 may have a dominant negative effect (Figure 5A, third panel). To examine Flag:YB-1 and Flag:YB-1A102 localization at the cellular level, immunofluorescence was performed in collaboration with Min Chen (PhD candidate, Dr. Catherine Pallen's Lab). Again, under log-growing conditions Flag:YB-1 was detected both in the cytoplasm and the nucleus (Figure 5B, middle panel). Nonetheless, Flag:YB-1A102 was predominantly found in the cytoplasm (Figure 5B, bottom panel). The staining from Flag antibody appeared to be specific since only a small amount of non-specific signal on the plasma membrane, but not throughout the cytoplasm or the nucleus, was observed in MCF-7 cells expressing the Flag empty vector (Flag:EV; Figure 5B, top panel). Interestingly, while the results from western blotting demonstrated an equal distribution of Flag:YB-1 in the cytoplasm and the nucleus (Figure 5A, second panel), the data obtained from immunofluorescence showed that there were more Flag:YB-1 in the cytoplasm (Figure 5B, middle panel). The results given by western blotting should be analyzed with caution. For western blotting, 50 μ g of total cytoplasmic or nucleic proteins were loaded. However, the amounts of proteins in the cytoplasm and the nucleus in a living cell are not necessarily the same. The levels of the cytoplasmic and nuclear Flag:YB-1 detected by western blotting may not reflect the actual protein distribution in a single cell. In conclusion, data obtained by both techniques demonstrated that mutation of Ser102 affected the cytoplasmic-nuclear shuttling of YB-1 under log growing conditions.

3.2 Activated YB-1 Induces EGFR Expression

3.2.1 YB-1 Promotes EGFR mRNA and Protein Expression

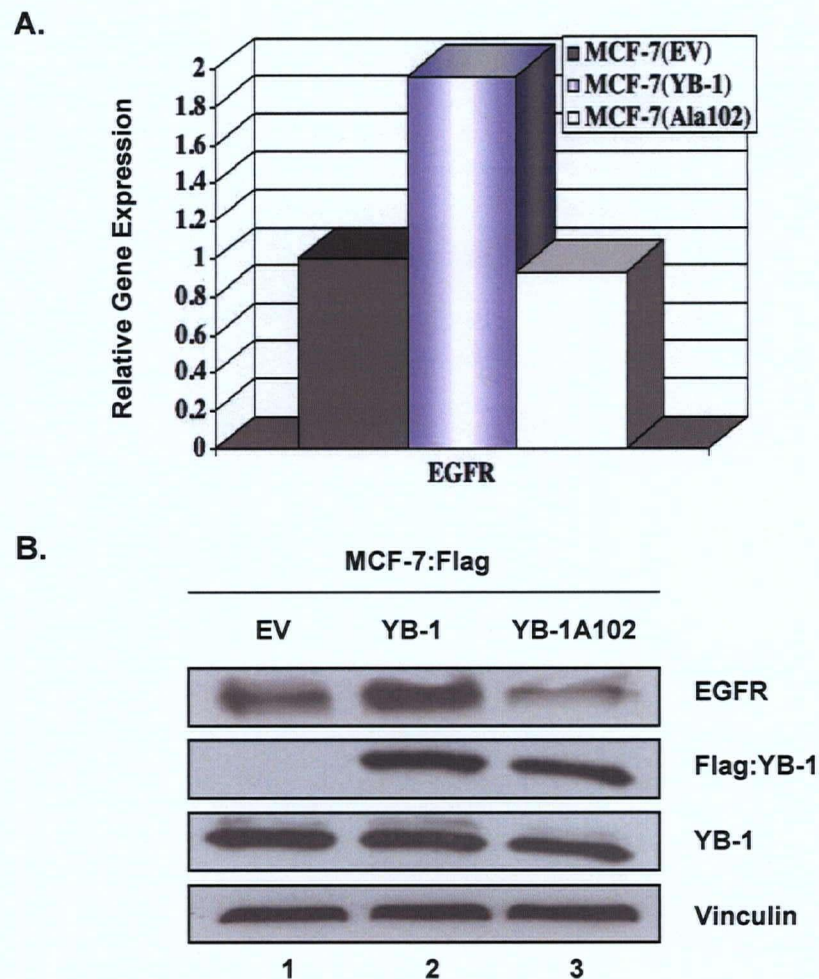
Having shown that YB-1 is regulated via the PI3K/Akt signaling, we next investigated the downstream effector of activated YB-1. Our lab has recently demonstrated that YB-1, but not YB-1A102, enhanced breast cancer cell growth both in monolayer and anchorage-independent conditions (Sutherland *et. al.* 2005). Since YB-1 was originally isolated as a DNA binding protein that interacts with the regulatory elements of *EGFR* (Sakura *et. al.* 1989), we revisited the possibility that YB-1 promotes breast cancer cell growth by inducing the expression of EGFR. MCF-7 cells were again chosen as a model system because they express low level of EGFR (Figure 3, lane 3), which enabled us to compare the effect of exogenous YB-1 and YB-1A102 on EGFR expression. In comparison with Flag:EV, the levels of EGFR mRNA and protein were increased by the expression of Flag:YB-1 in cells (Figure 6A and Figure 6B, lanes 1 and 2). However, inductions of the EGFR mRNA and protein were not observed when the mutant Flag:YB-1A102 was present (Figure 6A). Interestingly, the level of EGFR proteins was even less in MCF-7 Flag:YB-1A102 compared to Flag:EV (Figure 6B, lanes 1 and 3), which was in agreement with previous observation that the mutant YB-1A102 may have a dominant negative effect on the endogenous YB-1 (section 3.1.2). The data shown in Figure 5A and 5B were obtained in courtesy of Dr. Sandra Dunn and Daniel Yokom (summer student, Dr. Sandra Dunn's lab), respectively.

3.2.2 YB-1 Interacts with the Promoter of *EGFR*

The data from section 3.2.1 suggested that YB-1 regulates EGFR expression in part, if not all, at the transcriptional level. We have identified several putative YB-1 responsive elements (YREs) that contain the inverted CCAAT boxes (ATTGG or ATTG; Figure 7), by retrieving the

Figure 6²**YB-1, but not YB-1A102, Induced EGFR Expression**

(A) The level of EGFR mRNA transcript was measured by qRT-PCR in MCF-7 cells expressing Flag:EV, Flag:YB-1, or Flag:YB-1A102. The amount of EGFR mRNA in MCF-Flag:EV was considered as the basal-level and was set as "1". qRT-PCR was performed by Dr. Sandra Dunn and presented here with permission. (B) The amount of EGFR protein in MCF-7 expressing Flag:EV, Flag:YB-1, or Flag:YB-1A102 was measured by western blotting. Vinculin was used as loading control. This western blotting was performed by Daniel Yokom (summer student, Dr. Sandra Dunn's lab) and presented here with permission.

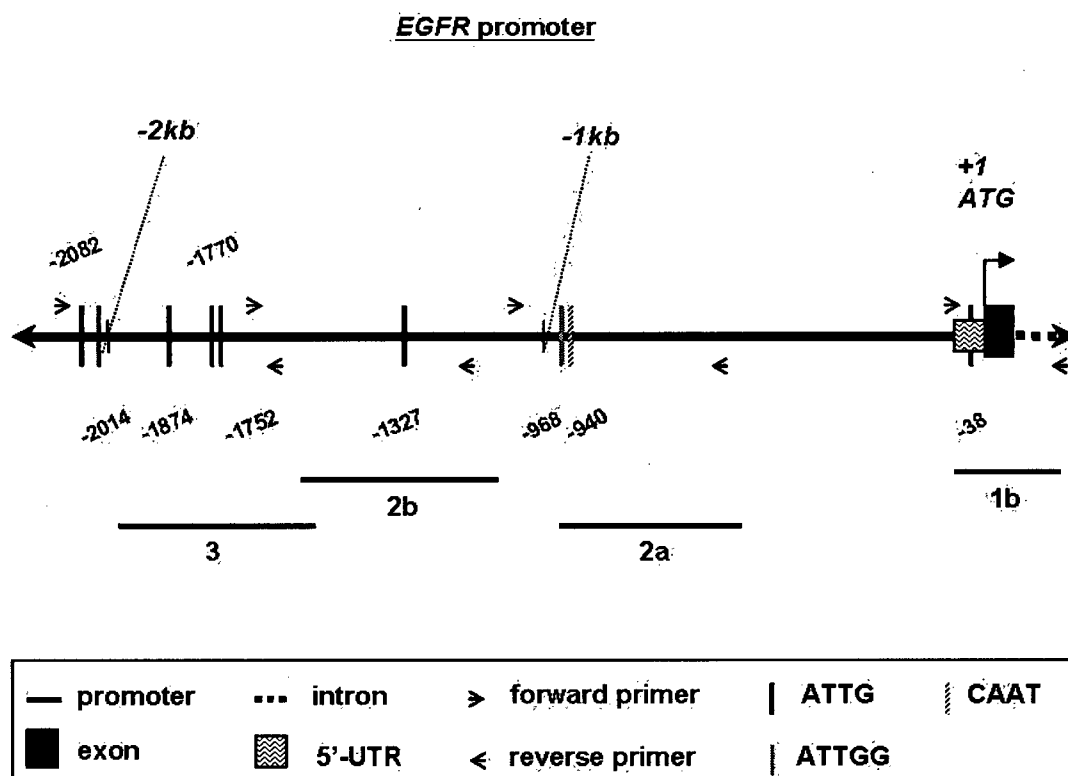


²Published in Wu *et. al. Cancer Res.* 2006.

-2 kb *EGFR* promoter sequence from the UCSD genome browser (www.genome.ucsc.edu/). In addition, YB-1 has been shown to interact with the antisense strand of the DNA (McDonald *et. al.* 1995). Therefore we also scanned the -2 kb of the reverse strand of the *EGFR* promoter for inverted CCAAT boxes. In total, nine putative YREs were found in the -2 kb of the *EGFR* promoter. While the occurrence of these YREs may be random, it is possible that these YREs are functional in regulating *EGFR* transcription. Chromatin-immunoprecipitation (ChIP) was therefore performed to first investigate whether there is an interaction between YB-1 and the *EGFR* promoter. Since the CSD of YB-1 is known to bind to DNA, we also speculated whether disruption of Ser102 would effect YB-1:DNA binding and thus *EGFR* gene expression. Central to addressing this question, YB-1A102 was subjected to the ChIP assay as well. Four sets of primers were designed to analyze different regions containing the YREs in the -2 kb of *EGFR* promoter (Figure 7). The first 1 kb region was examined by primers 1b and 2a, while the region between -1 to -2 kb was investigated by primers 2b and 3. A primer set that amplified the *gapdh* gene was used as a negative control. Prior to ChIP, the efficiencies of the primers were assessed by amplifying genomic DNA from MCF-7 cells. All primer sets were able to amplify DNA templates producing PCR products of the expected sizes (Figure 8A). Next, chromatin from MCF-7 Flag:YB-1 or Flag:YB-1A102 was sheared into fragments between 200 bp and 1 kb (Figure 8B) by sonication, and protein:DNA complexes were isolated using the Flag antibody. To ensure that same amounts of input samples were used and that PCR was in the linear range, input DNAs from MCF-7 Flag:YB-1 and Flag:YB-1A102 were amplified and titrated with the 2a primers (Figure 8C). As shown in Figure 9A, Flag:YB-1 was able to interact with the first 2 kb of the *EGFR* promoter (Figure 9A, lanes 1-4). Intriguingly, while the interactions with the 2b and 3 region were retained (Figure 9A, lanes 8 and 9), binding to the 1b region by Flag:YB-1A102 was abolished (Figure 9A, lane 6). Also, Flag:YB-1A102 did not bind to the 2a region as strongly as

Figure 7³
The First 2 kb of the *EGFR* Promoter

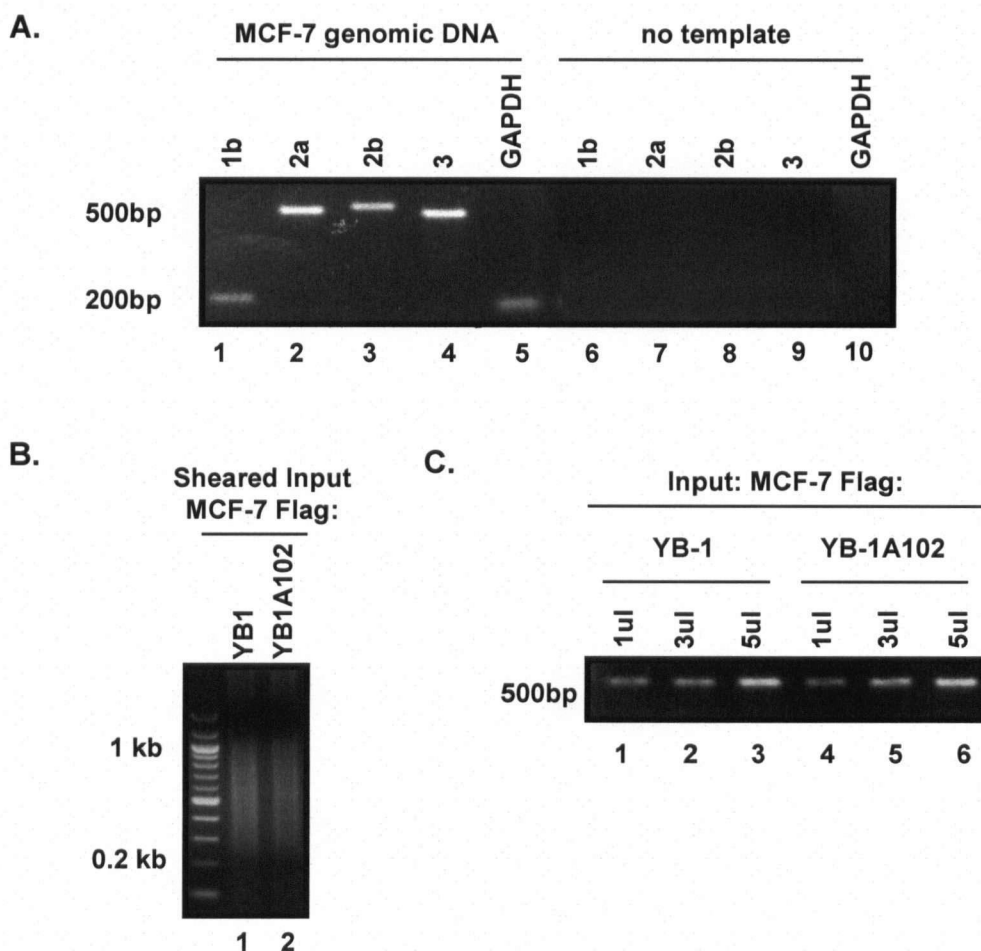
The first 2 kb of the *egfr* promoter was retrieved from the UCSD Genome Browser (<http://genome.ucsc.edu/>). Nine putative YREs were found, including one YRE in the 1b region, two YREs in the 2a region, one YRE in the 2b region, and 5 YREs in the 3 region. Four sets of primers (1b, 2a, 2b, and 3) were designed, and each primer set covers one or more YREs in the *egfr* promoter.



³Published in Wu *et. al. Cancer Res.* 2006.

Figure 8⁴**Validation of the Primers and Conditions for Chromatin-immunoprecipitation (ChIP)**

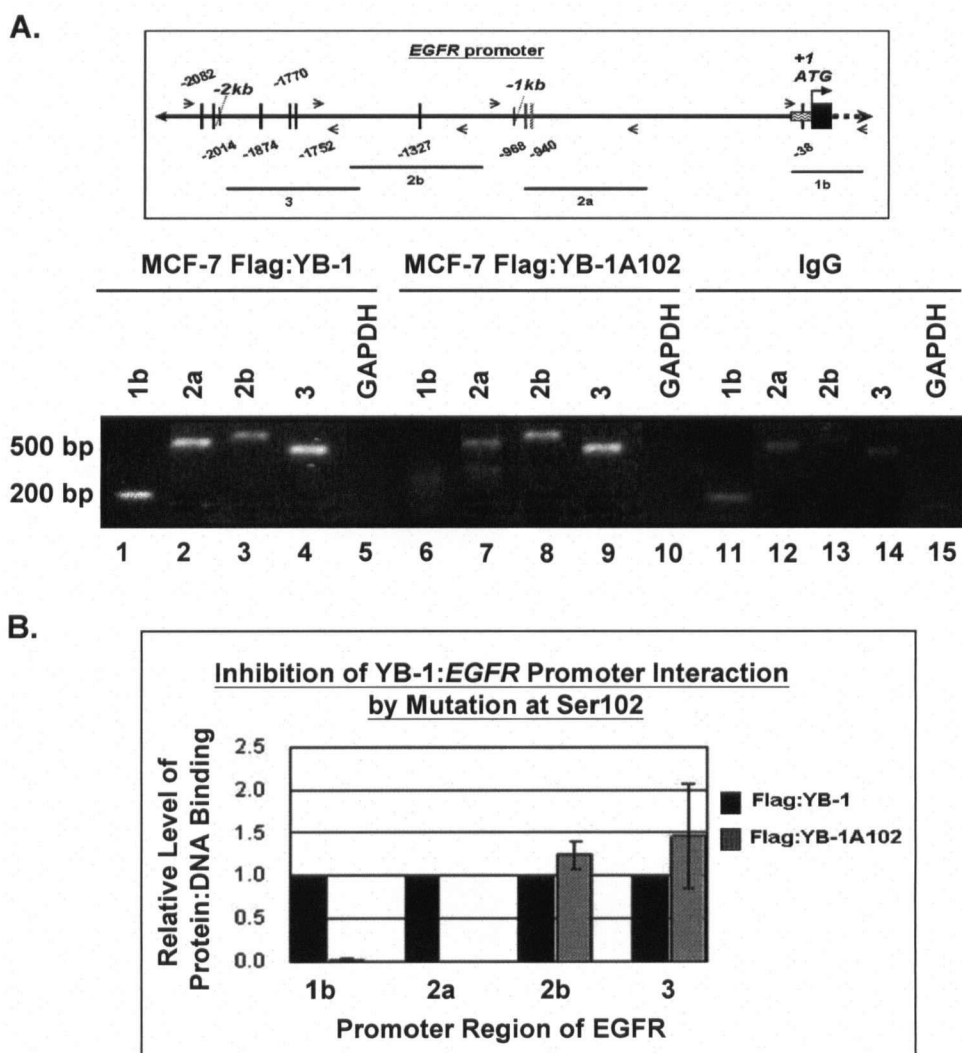
(A) Genomic DNA from MCF-7 cells was amplified using the EGFR primers (1b, 2a, 2b, and 3) as well as the GAPDH primers by PCR. The GAPDH primers were used as a negative control in the ChIP assay. The PCR products were analyzed on a 2% agarose gel. (B) Genomic DNA from MCF-7 expressing Flag:YB-1 or Flag:YB-1A102 was sheared by sonication. The sheared DNA was analyzed on a 2% agarose gel to confirm that the size of DNA fragments were between 200 bp and 1 kb. (C) Increasing amounts of input DNA from each sample was examined by PCR using the EGFR 2a primer set.



⁴Published in Wu *et. al. Cancer Res.* 2006.

Figure 9⁵YB-1, but not YB-1A102 Interacted with the -1 kb of the *EGFR* Promoter

(A) ChIP was performed in MCF-7 cells expressing Flag:YB-1 or Flag:YB-1A102. The DNA obtained from the ChIP assay was amplified using the four *EGFR* primer sets (1b, 2a, 2b, and 3) as well as the GAPDH primers and analyzed on a 2% agarose gel. IgG was used as a negative control for the ChIP Assay. (B) The assay was repeated three times, and the intensities of DNA bands were quantified using densitometry. The degree of Flag:YB-1 or Flag:YB-1A102 binding to each *EGFR* promoter region was calculated using the formula: (binding by YB-1/input)-(binding by IgG/input). The level of DNA binding by Flag:YB-1 was set as “1”.



⁵Published in Wu *et. al. Cancer Res.* 2006.

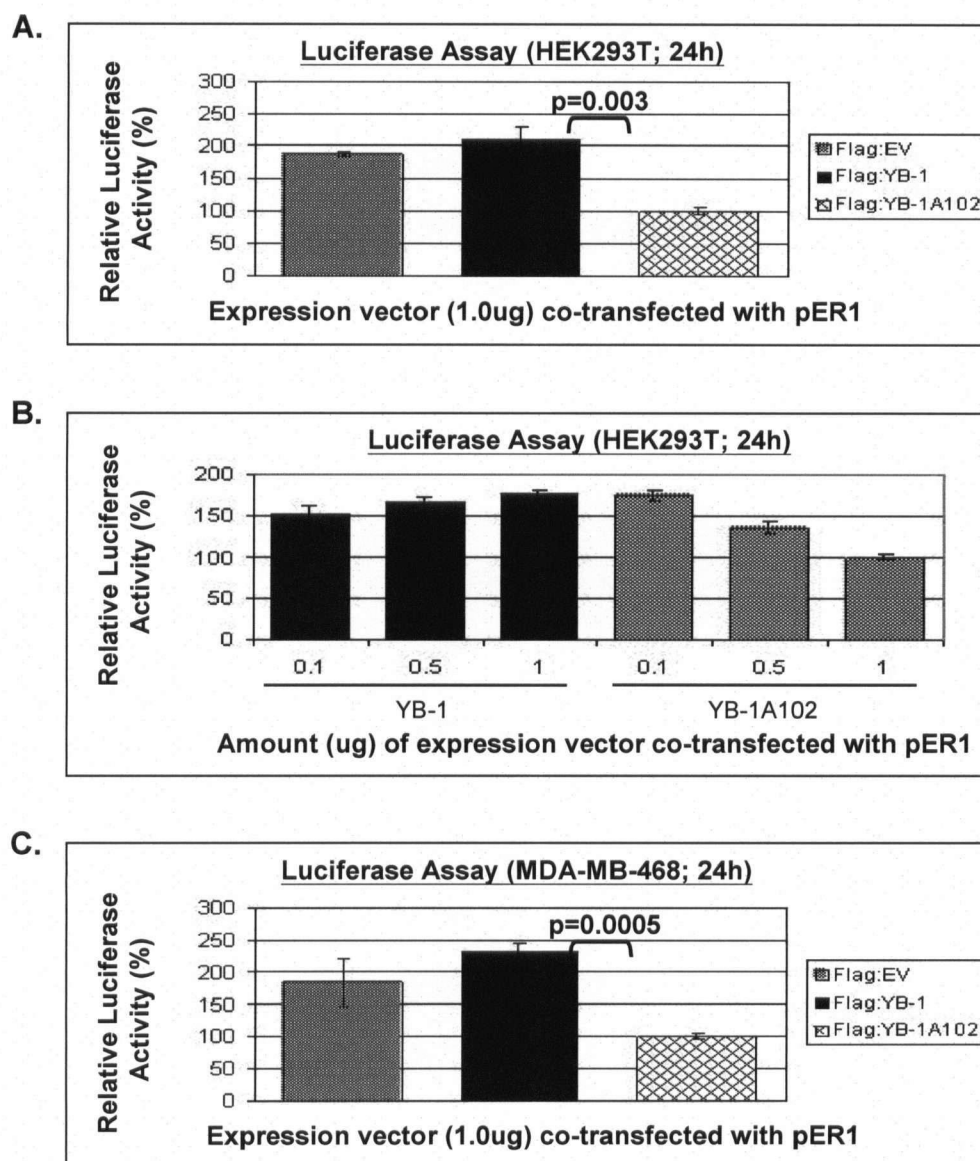
Flag:YB-1 did (Figure 9A, lane 7). Quantification of the intensities of the DNA bands revealed that while binding to the 2b and 3 regions by Flag:YB-1A102 was retained, disruption of Ser102 completely prevented YB-1 from interacting with the first 1 kb of the *EGFR* promoter (Figure 9B). The inability of Flag:YB-1A102 to bind to the -1 kb region may be the reason why Flag:YB-1A102 was not capable of inducing *EGFR* gene expression (section 3.2.1). Furthermore, although a small amount of non-specific binding by IgG was observed (Figure 9A, lanes 11 to 15), there was no amplification by the GAPDH primers (Figure 9A, lanes 5 and 10). Thus, the binding of Flag:YB-1 or Flag:YB-1A102 to the *EGFR* promoter was considered specific. Taken together, we concluded that Ser102 is involved in DNA binding. Specifically, YB-1 interacts with the *EGFR* promoter, and Ser102 is required for optimal binding to the -1 kb region.

3.2.3 YB-1 Activates the Promoter of *EGFR*

The ability of Flag:YB-1, but not Flag:YB-1A102, to bind to the -1 kb of the *EGFR* promoter suggested that YB-1 may regulate *EGFR* transcription via this region. To address this possibility, reporter assays were performed using a luciferase construct (pER1) harboring the -16 to -1109 sequence of the *EGFR* promoter, which was a generous gift from Dr. Alfred Johnson from Maryland, U.S. The construct was first transfected in MCF-7 expressing Flag:EV, Flag:YB-1, or Flag:YB-1A102. Unfortunately, we could hardly detect luciferase activity in any of these cell lines (data not shown). One reason could be that since MCF-7 cells are estrogen receptor positive (ER(+)) and express very little EGFR (Figure 3, lane 3), there could be strong repressors acting on the EGFR promoter in this cell line. Besides, phosphorylation of YB-1 may be minimal as Akt is not activated in MCF-7 cells. We also learned that luciferase activity is relatively hard to measure to produce significant and consistent data in MCF-7 possibly due to the nature and the low transfection efficiency of the cell line (personal communication with Dr.

Figure 10
Disruption of Ser102 Inhibited YB-1 to Activate *EGFR* Transcription

Luciferase Assays were performed by co-transfecting pER1 with either (A) 1 μ g of the expression vector in HEK293T, (B) increasing amounts (0.1, 0.5, and 1 μ g) of the expression vector in HEK293T cells, or (C) 1 μ g of the expression vector in MDA-MB-468 cells. Each assay was performed in triplicates and repeated three times. The luciferase activity measured from cells containing 1 μ g of the Flag YB-1A102 expression vector was set as "100%". A student t-test was performed to calculate the p-value.



Catherine Pallen). As a result, we changed the model system to the human embryonic kidney 293 (HEK293T) cells, as they are easier to transfect. After transient co-transfection of pER1 with the Flag:YB-1 expression vector or the empty vector, no induction or very small induction of the luciferase activity was observed (Figure 10A). However, luciferase activity was significantly lower in cells co-transfected with the Flag:YB-1A102 mutant ($p=0.003$; Figure 10A). The decrease in luciferase activity by Flag:YB-1A102 was also dose-dependent (Figure 10B). To examine whether the result was reproducible in a breast cancer cell line, luciferase assay was performed in MDA-MB-468 breast cancer cells. This cell line was chosen because it expresses high levels of EGFR and activated Akt (Figure 3, lane 4). Consistently, we did not observe a significant increase in luciferase activity in the presence of the Flag:YB-1 expression vector (Figure 10C). However, Flag:YB-1A102 was again able to significantly suppress the reporter activity ($p=0.0005$; Figure 10C). The inability of Flag:YB-1 to further induce EGFR promoter activity in neither HEK293T nor MDA-MB-468 cells may be due to intrinsically high levels of YB-1 and pAkt in these cell lines (Figure 3, lanes 2 and 4). Together, these data demonstrated that when Ser102 was mutated, YB-1 lost its ability to activate the -1 kb of the *EGFR* promoter.

3.3 OSU-03012 Inhibits the Functions of YB-1

3.3.1 OSU-03012 Blocks YB-1 Nuclear Translocation

For the past few years, our lab has been interested in identifying small molecular inhibitors for treating breast cancer. Recently, we have demonstrated that the celecoxib derivative, OSU-03012, a stable and orally available potent PDK1 specific inhibitor, decreased the growth of breast cancer cells with high level of pAkt (Kucab *et. al.* 2005). Having demonstrated YB-1 as a downstream component of PI3K/Akt pathway, it is therefore possible to inhibit the activity of YB-1 using OSU-03012. First, we examined whether OSU-03012 prevented YB-1 from

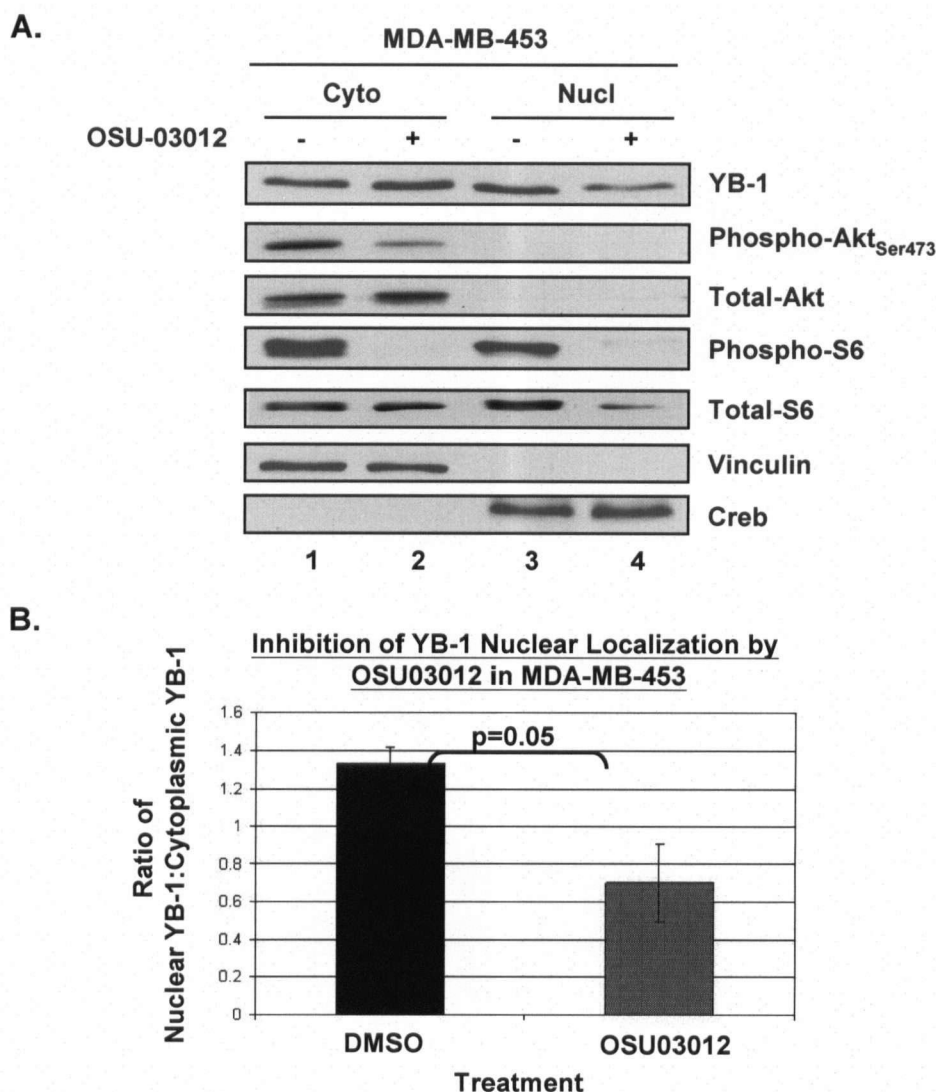
translocating into the nucleus. The MDA-MB-453 breast cancer cells in which Akt was highly activated (Noh *et al.* 2004) were treated with OSU-03012 for 6 hour, and YB-1 nuclear trafficking was inspected by cytoplasmic-nuclear fractionation and western blotting. Under normal log growing conditions, YB-1 was detected both in the cytoplasm and the nucleus (Figure 11A, lanes 1 and 3). However, when log-phase proliferating cells were treated with OSU-03012, the level of nuclear and cytoplasmic YB-1 decreased and increased, respectively (Figure 11A, lanes 2 and 4). In fact, the ratio of nuclear to cytoplasmic YB-1 decreased almost 2 fold in OSU-03012 treated cells ($p=0.05$; Figure 11B). The efficacy of the drug to inhibit PDK1 signaling was assessed by a decrease in the phosphorylation of Akt and the S6 ribosomal protein (Figure 11A, panel 2 and 4). It was noted that the level of total S6 ribosomal protein changed after drug treatment. In fact, we and others have observed inconsistent levels of total S6 ribosomal protein after drug treatment or growth factor stimulation. Nonetheless, the phosphorylation of S6 ribosomal protein is still widely used by researchers as a downstream marker of the PI3K/Akt signaling. The levels of vinculin and creb were not affected by OSU-03012 (Figure 11A, panels 6 and 7), indicating that decrease in YB-1 protein level by OSU-03012 was specific.

3.3.2 OSU-03012 Prevented YB-1 from Binding to the *EGFR* Promoter

Since it was determined that disruption of Ser102 prevented YB-1 from binding to the -1 kb of the *EGFR* promoter as detected by ChIP using the 1b and 2a primer sets, we then asked if the interaction between YB-1 and the -1 kb of *EGFR* promoter can be abolished by OSU03012. MDA-MB-468 cells provided a good model system for this study because they express high levels of pAkt and EGFR (Figure 3, lane 4). MDA-MB-468 cells were treated with OSU03012 for 6 hours, and the effect of the drug was indicated by decreases in the levels of pAkt and pS6

Figure 11
OSU-03012 Inhibited YB-1 Nuclear Translocation

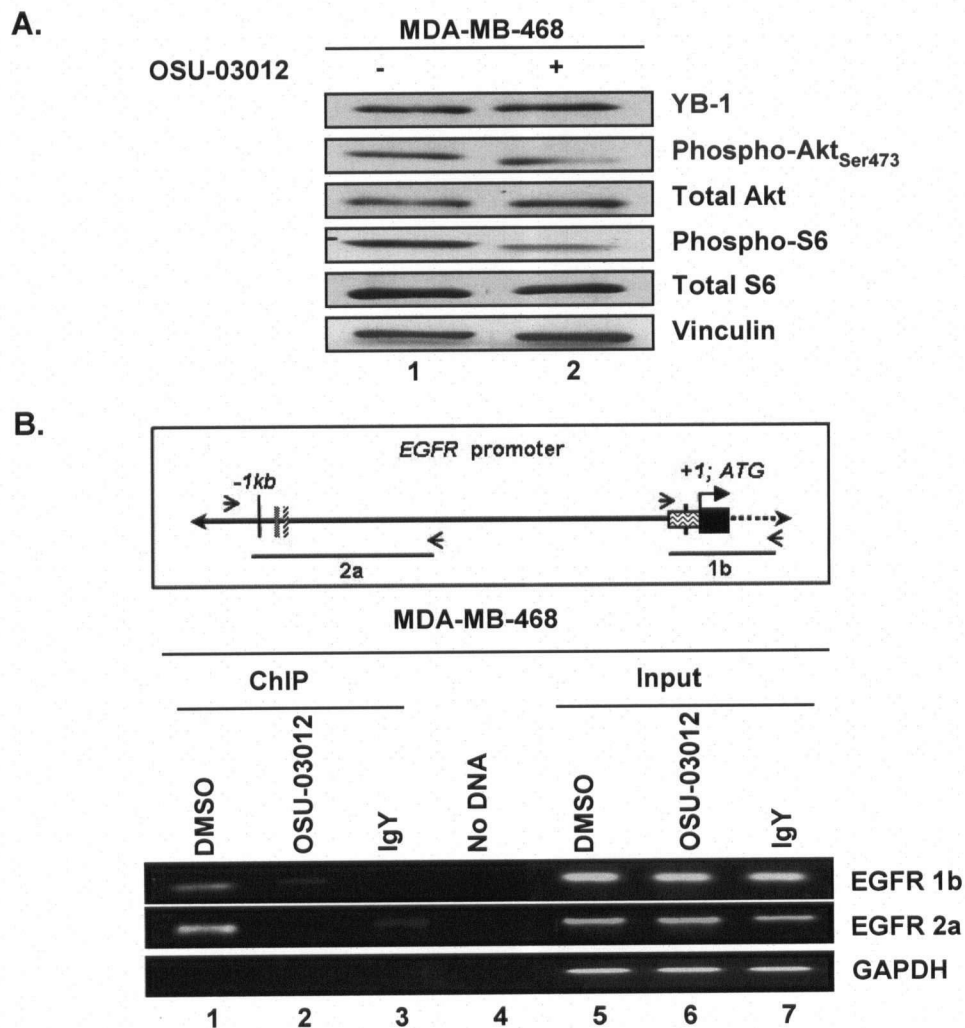
(A) MDA-MB-453 cells were treated with OSU-03012, and cytoplasmic and nuclear protein fractions were extracted. The cellular localization of YB-1 was examined by western blotting. Vinculin and creb were used as loading controls for cytoplasmic and nuclear fractions, respectively. (B) The experiment was repeated three times, and the band intensities of cytoplasmic and nuclear YB-1 were measured by densitometry. The difference in YB-1 cellular localization upon OSU-03012 treatment was assessed by the ratio of (nuclear YB-1/cytoplasmic YB-1). A student t-test was performed to calculate the p-value.



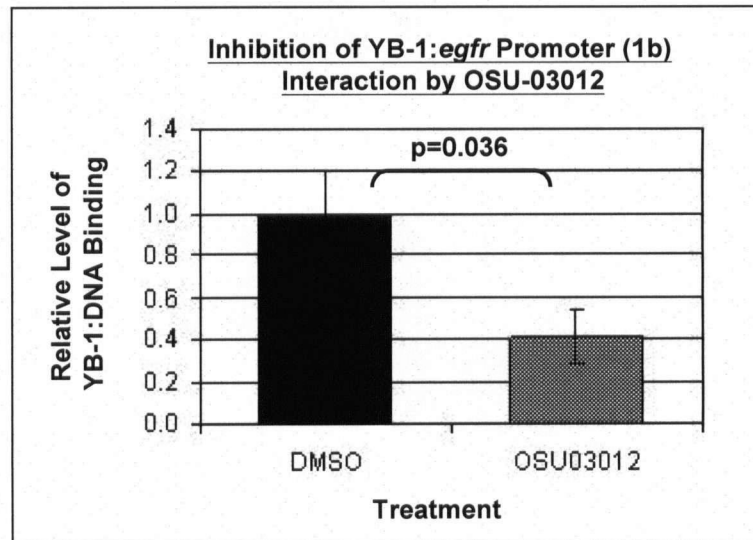
ribosomal protein (Figure 12A, panels 2 and 4). ChIP was performed and there was a clear loss of binding of YB-1 to the first 1 kb of the *EGFR* promoter after treatment with OSU-03012 (Figure 12B, top and middle panels). The reduced binding of YB-1 to the *EGFR* promoter in OSU03012-treated cells was not due to unequal inputs (Figure 12B, lanes 5 to 7). Furthermore, the specificity of the ChIP assay was confirmed as there was no non-specific binding by IgY (Figure 12B, lane 3) and DNA was not amplified by the GAPDH primers (Figure 12B, bottom panel). The ability of OSU-03012 to inhibit the binding of YB-1 to the 1b and 2a regions was quantified using densitometry. Interestingly, while the binding of YB-1 to the 1b region was prevented more than 2 fold ($p=0.036$; Figure 12C), the interaction between YB-1 and the 2a region was almost completely abrogated ($p=0.024$; Figure 12D). Whether the interaction between YB-1 and 2b and 3 is also affected by OSU-03012 is currently under investigation. In summary, OSU-03012 was able to block YB-1 to shuttle into the nucleus and to bind to the *EGFR* promoter. Moreover, the data generated here further verified the relationship between Akt, YB-1, and EGFR.

Figure 12**OSU-03012 Prevented YB-1 from Binding to the -1 kb of the *EGFR* Promoter**

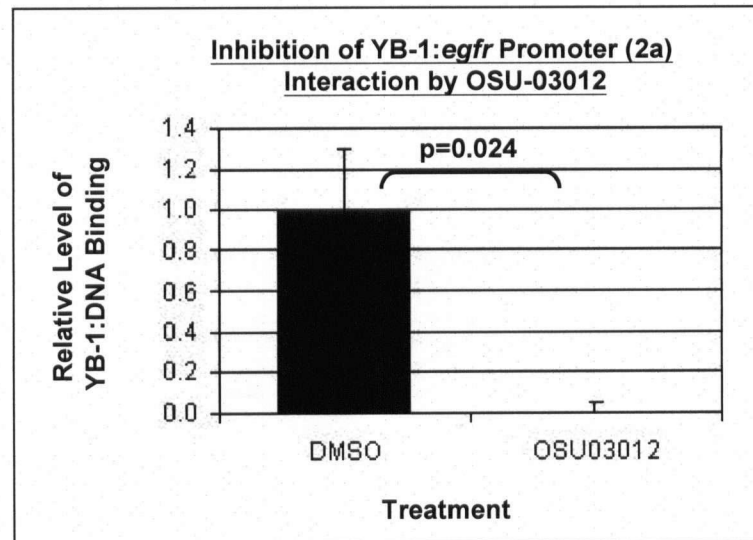
(A) MDA-MB-468 cells were treated with OSU-03012. The cell lysates were examined by western blot to ensure that OSU-03012 inhibited the PI3K/Akt signaling. Vinculin was used as a loading control. (B) DNA of untreated or OSU-03012 treated cells obtained from ChIP assays, which were repeated three times, were amplified using the *EGFR* 1b or 2a primers. The PCR products were analyzed on a 2% agarose gel. IgY was used as a negative control for the ChIP assays. The intensities of DNA bands were quantified using densitometry. The degree of YB-1 binding to the (C) *EGFR* 1b region or the (D) *EGFR* 2a region were calculated using the formula: $(\text{OSU-03012}_{\text{ChIP}}/\text{OSU-03012}_{\text{Input}}) - (\text{IgY}_{\text{ChIP}}/\text{IgY}_{\text{Input}})$. The level of DNA binding by YB-1 in cells treated with DMSO was set as "1". A student t-test was performed to calculate the p-value.



C.



D.



CHAPTER 4

DISCUSSION

The importances of Akt, YB-1, and EGFR have been separately implicated in breast cancer. In this study, we have demonstrated a functional link that connects all three proteins in the same pathway. We first showed that YB-1 is phosphorylated at serine residues through PI3K/Akt signaling in the context of breast cancer cells by immunoprecipitation and western blotting (section 3.1.1). Since the CSD has been suggested to contribute to YB-1 cellular localization, we then assessed whether Ser102 may be involved in YB-1 nuclear translocation. We found that disruption of Ser102 prevented YB-1 from moving into the nucleus by western blotting and immunofluorescence (section 3.1.2). We next investigated the possibility that YB-1 up-regulates the expression of EGFR. It was discovered that YB-1 but not YB-1A102 increased both the EGFR mRNA and protein levels (section 3.2.1). This result suggested that YB-1 may regulate *EGFR* transcription. To assess whether this was the case, we determined if YB-1 bound the *EGFR* promoter. We first identified nine putative YREs in the -2 kb of the *EGFR* promoter. Results from chromatin immunoprecipitation revealed that while YB-1 interacted with the -2 kb of the *EGFR* promoter, YB-1A102 could only bind to the -1 kb region (section 3.2.2). By using luciferase reporter assays, we discovered that transfection of the Flag:YB-1 expression vector could only activate the *EGFR* promoter to a small extent. However, Flag:YB-1A102 suppressed *EGFR* transcription significantly (section 3.2.3). Together, these data support the rationale that PI3K/Akt activates YB-1, which in turn stimulates EGFR expression resulting in enhanced cancer cell growth. Finally, we used OSU-03012 to target PDK1 and thus Akt to further validate the association among Akt, YB-1, and EGFR. It was found that OSU-03012 inhibited YB-1

nuclear localization and binding to the *EGFR* promoter (section 3.3). In light of the work presented here, YB-1 may be considered as a novel therapeutic target to treat breast cancer.

4.1 Phosphorylation of YB-1 by PI3K/Akt

In this study, we first demonstrated that YB-1 is a component of the PI3K/Akt pathway. Upon Akt activation by IGF-1 stimulation, YB-1 is phosphorylated at serine residues *ex vivo*. It is interesting to note that the location of Ser102 in the CSD is in a flexible loop region of YB-1, which is accessible to the crevice formed by Akt1 in its active conformation (Sutherland *et. al.* 2005). In this experiment we observed that pYB-1 migrated slower than YB-1 on a SDS-PAGE gel. Phosphorylation is known to affect protein mobility on a gel. Taking FKHRL1 as an example, when it is phosphorylated by Akt, a band shift was observed (Biggs *et. al.* 1999 or Brunet *et. al.* 1999). The mechanism for band shifting is not clear but is thought to involve the negative charge of the phosphate group, which interferes with SDS to bind to the protein.

It is possible that the single band detected by the phospho-serine antibody did not represent pYB-1 as other proteins might be co-immunoprecipitated with YB-1. To further confirm that the up-shifting band is indeed pYB-1, phosphatase treatment, which should cause the up-shifting band to disappear, can be performed. Alternatively, the protein can be extracted from the gel and sequenced by mass spectrophotometry. The advantage of the second approach is that other phosphorylation sites on YB-1 can be discovered. Indeed, evidence suggests that Akt is not the only protein to phosphorylate YB-1, which is discussed below.

4.1.1 Potential Proteins that Phosphorylate YB-1 via PI3K Signaling

This year Evdokimova *et. al.* similarly demonstrated that IGF-1 stimulation leads to YB-1 phosphorylation. In their study, YB-1 was immunoprecipitated with a custom-made YB-1

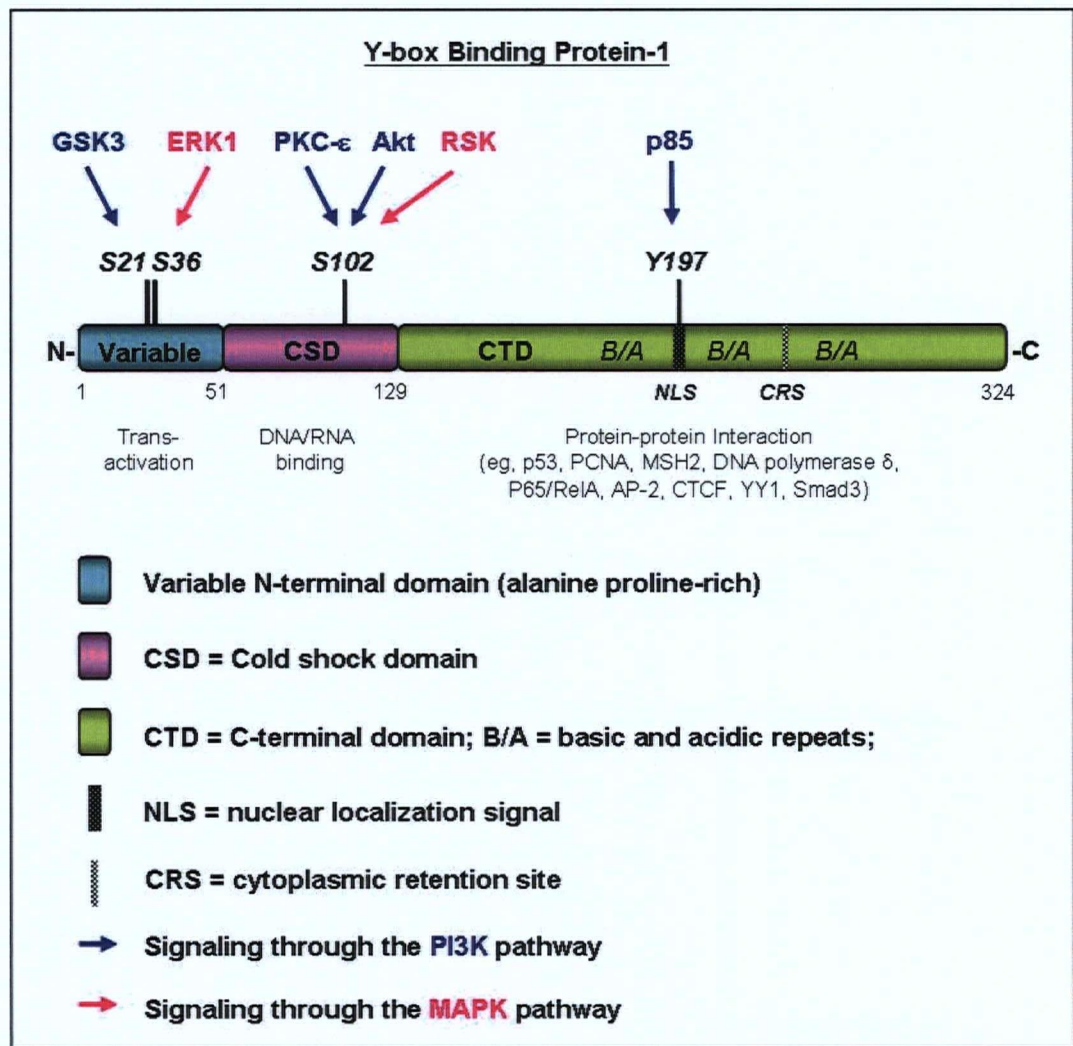
antibody, and phosphorylation of YB-1 was detected by radio-labeled orthophosphate (Evdokimova *et. al.* 2006). Wortmannin, which is another PI3K inhibitor, fully blocked phosphorylation of YB-1. However, mutating Ser102 to alanine did not completely prevent YB-1 phosphorylation, suggesting that other components of PI3K/Akt cascade may be involved (Evdokimova *et. al.* 2006). To explore this possibility, we used the motif scanner prediction tool (<http://scansite.mit.edu/motifscanner/motifscan2.html>) to identify putative YB-1 phosphorylation sites (Figure 13). Interestingly, protein kinase C (PKC)- ϵ , a PKC isoform characterized as a calcium-independent and phorbol ester/diacylglycerol-sensitive serine/threonine kinase, could potentially phosphorylate YB-1 at Ser102. It has been shown that PI3K converts PIP2 to PIP3, which activates PKC- ϵ , resulting in cell proliferation and survival (Moriya *et. al.* 1996, Graness *et. al.* 1998). PKC- ϵ has been implicated in tumorigenesis, invasion, and metastasis (Mischak *et. al.* 1993, Perletti *et. al.* 1998, Wu *et. al.* 2002, Jansen *et. al.* 2001, Berrier *et. al.* 2000). The role of PKC- ϵ in breast cancer is less defined, and whether phosphorylation of YB-1 at Ser102 by PKC- ϵ contributes to the pathogenesis needs further investigation. Moreover, the glycogen synthase kinase 3 (GSK3), which is negatively regulated by Akt (Diehl *et. al.* 1998; section 1.2.2), could also potentially phosphorylate YB-1 at Ser21 in the N-terminal domain and affect the transcriptional activity of YB-1 (Figure 13). GSK3 has been implicated in cancers as it is involved in the degradation of β -catenin, a marker of the epithelial-mesenchymal-transition (EMT) and metastasis (Aberle *et. al.* 1997). It is therefore motivating to examine whether GSK3 phosphorylates YB-1 and whether this event has any significance in breast cancer progression.

Furthermore, Since Evdokimova *et. al.* demonstrated YB-1 phosphorylation via the PI3K/Akt signaling in cells by using radioactive ATP (Evdokimova *et. al.* 2006), phosphorylation of YB-1 detected by this approach could also be due to phosphorylation at residues other than serines. Indeed, results from the motif scanning prediction tool suggested that the p85 subunit of

Figure 13

Putative Proteins that Phosphorylate YB-1 via the PI3K or the MAPK Signaling Pathway

To examine possible kinases that could also phosphorylate YB-1 through the PI3K or the MAPK signaling pathway, the motif scanning prediction tool (<http://scansite.mit.edu/motifscanner/motifscan2.html>) was used. It was found that in addition to Akt, PKC- ϵ may also phosphorylate YB-1 at Ser102. Since RSK recognizes the same sequence as Akt in its binding partners, RSK is another potential kinase to phosphorylate YB-1 at Ser102. GSK3, ERK1, and the p85 subunit of PI3K were also suggested by the motif scanning prediction tool to phosphorylate YB-1 at Ser21, Ser36, and Tyr197, respectively.



PI3K may phosphorylate YB-1 at Tyr197 in the C-terminal domain (Figure 13). Curiously, Tyr197 is located within the NLS (residue 183-202; Bader *et. al.* 2005) of YB-1, which raises the question whether YB-1 subcellular localization is affected by phosphorylation at Tyr197 by PI3K. If YB-1 is phosphorylated by one or more proteins discussed above, inhibiting the PI3K signaling by Ly294002 or wortmannin should block YB-1 phosphorylation via PI3K signaling. Mutation of Ser102, however, will not completely inhibit YB-1 phosphorylation, as observed by Evdokimova and coworkers (Evdokimova *et. al.* 2006).

4.1.2 Potential Proteins that Phosphorylate YB-1 via MAPK Signaling

The possibility that YB-1 may be phosphorylated by other signaling pathways cannot be ruled out. Our lab has recently discovered that neither Ly294002 nor OSU-03012 decreased YB-1 serine phosphorylation in aggressive breast cancer cell lines such as MDA-MB-453 and MDA-MB-468 (data not shown). In these cell lines, both PI3K/Akt and MAPK pathways are highly activated. Thus, it is possible that the MAPK pathway also contributes to YB-1 serine phosphorylation. In support of this hypothesis, data obtained from the motif scanning prediction tool suggested a few players of the MAPK/ERK pathways as putative proteins to phosphorylate YB-1 at serine residues (Figure 13). For example, the Ser36 residue located in the transactivating N-terminal domain of YB-1 may be a target for the extracellular-signal-regulated kinase 1 (ERK1). ERK is activated by MAPK in response to growth factor stimulation, resulting in cell proliferation and survival (Chang and Karin 2001, Sebolt-Leopold and Herrera 2004). Elevated MAPK/ERK signaling also leads to malignancies such as breast cancer (Sivaraman *et. al.* 1997, El-Ashry *et. al.* 1997, Coutts and Murphy 1998, Donovan *et. al.* 2001). It would be interesting to pursue whether YB-1 and the MAPK/ERK pathway work cooperatively in breast cancer pathogenesis. Small molecules such as the MEK inhibitor, PD98059, would be valuable in

determining the role of YB-1 in the MAPK/ERK pathway in breast cancer cells. Finally, Akt binds to and phosphorylates YB-1 by recognizing the RxRxxS/T motif, in which S is the S102 residue (Alessi *et. al.* 1996). It was noted that the p90 ribosomal S6 kinase (RSK), a downstream component of MAPK/ERK signaling (Frodin and Gammeltoft 1999), also recognize the same sequence in its binding partner (Alessi *et. al.* 1996). Therefore, RSK could be another candidate protein that activates YB-1 (Figure 13).

4.2 The Role of Ser102 in the Regulation of YB-1 by Akt

We have examined the importance of Ser102 in the regulation of YB-1 by Akt by using the YB-1A102 mutant. It was revealed that Ser102 is involved in YB-1 nuclear localization and DNA binding, which ultimately influence the ability of YB-1 to activate transcription.

4.2.1 Ser102 and Nuclear Translocation

The disruption of Ser102 retained Flag:YB-1A102 in the cytoplasm (section 3.1.2). Besides, treatment of OSU-03012 prevented endogenous YB-1 to traffic into the nucleus, resulting in the accumulation of YB-1 in the cytoplasm (section 3.3.2). Therefore, nuclear translocation of YB-1 can be controlled by Ser102 phosphorylation via the PI3K/Akt pathway. To further confirm that activation of PI3K/Akt pathway leads to YB-1 nuclear localization, we have previously stimulated the cells with IGF-1 for 1 hour and examined YB-1 cellular localization by western blotting. Treatment of IGF-1 for 1 hour did not induce YB-1 nuclear translocation (data not shown). In a study conducted by Higashi *et. al.*, the ability of interferon- γ (IFN- γ) to promote YB-1 nuclear translocation was demonstrated (Higashi *et. al.* 2003). Interestingly, IFN- γ induced nuclear translocation of YB-1 was observed 6 hours after stimulation (Higashi *et. al.* 2003). Thus, it is possible that stimulation of IGF-1 for 1 hour may

not be long enough to induce YB-1 nuclear translocation.

Akt is known to directly or indirectly control the cytoplasmic-nuclear localization of proteins, or transcription factors, through different mechanisms. First of all, Akt can phosphorylate the nuclear localization signal (NLS) or the nuclear export signal (NES) of its substrates, such as the FOXO forkhead transcription factors and p27. In the case of FOXO, hyperphosphorylation of FOXO proteins by Akt at NLS and NES facilitates binding with 14-3-3, which promotes the nuclear export of FOXO proteins through the exportin CRM1 (Brunet *et. al.* 2002, Rena *et. al.* 2001, Biggs *et. al.* 1999, Brownawell *et. al.* 2001). On the other hand, Akt phosphorylation at the NLS of p27 inhibits p27 nuclear import resulting in cytoplasmic sequestration (Liang *et. al.* 2002, Shin *et. al.* 2002). Recently, YB-1 was shown to have a noncanonical NLS as well as a cytoplasmic retention site (CRS) that prevails over the NLS (Bader *et. al.* 2005). However, both of NLS and CRS are located in the C-terminal domain of YB-1 whereas Ser102 is resided in the CSD. Therefore, it is unlikely that Akt regulates YB-1 cellular localization directly through NLS or CRS phosphorylation. Nonetheless, it is possible that Ser102 cooperates with NLS and/or CRS in YB-1 nuclear trafficking. In fact, it has been suggested by Jürchott *et. al.* that both the C-terminus and the CSD are involved in YB-1 nuclear shuttling (Jürchott *et. al.* 2003).

Another approach utilized by Akt to affect protein cellular localization involves the phosphorylation of an inhibitor that is bound to the NLS of the protein. For instance, under basal condition the transcription factor NF- κ B forms a complex with the inhibitor I κ B, which masks the NLS on NF- κ B and prevents translocation of NF- κ B to the nucleus. Upon growth factor stimulation, Akt becomes phosphorylated and activates the I κ B kinase (IKK). The activated IKK in turn phosphorylates I κ B and targets it for degradation by the 26S proteasome. Consequently, the NLS is revealed and NF- κ B nuclear import occurs (Henkel *et. al.* 1992, Beg *et. al.* 1992). To

date, no proteins that bind the NLS or CRS of YB-1 have been found. Although it has been reported that functional p53 is required for YB-1 nuclear translocation, physical interaction between the two proteins is not involved (Zhang *et. al.* 2003). It was suggested that p53 causes nuclear translocation of YB-1 by activating a downstream target gene, the product of which mediates this translocation process (Zhang *et. al.* 2003). In normal cells, YB-1 mainly locates in the cytoplasm, where it is bound to mRNA. As illustrated by Stenina *et. al.*, the release of YB-1 from mRNA induced by PDGF and the thrombin protease results in YB-1 nuclear localization (Stenina *et. al.* 2001). It was further demonstrated that phosphorylation of YB-1 at Ser102 by Akt reduced the interaction of YB-1 with the capped 5' terminus of mRNA (Evdokimova *et. al.* 2006). Therefore, the binding of YB-1 to mRNA may be analogous to the interaction of NF- κ B with I κ B in the cytoplasm, the effect of both is the prevention of transcription factors to translocate into the nucleus.

Taken together, we propose a model that may explain how YB-1 cellular trafficking occurs. In unstimulated cells, YB-1 mainly resides in the cytoplasm where it is bound to mRNA. The association between YB-1 and mRNA may prevent YB-1 from degradation. In support of this view, it has been demonstrated that proteasome cleavage of YB-1 is protected by mRNA binding (Sorokin *et. al.* 2005). Upon stimulation by growth factors, PI3K/Akt signaling phosphorylates YB-1 at Ser102 located in CSD, resulting in the dissociation of YB-1 from mRNA in the cytoplasm. Furthermore, phosphorylation of Ser102 may also induce a conformational change in YB-1, which may uncover NLS and/or mask CRS. Consequently, YB-1 can be transported into the nucleus through nuclear pores via the conventional NLS-importin α/β pathway. Once in the nucleus, YB-1 then binds to DNA and functions as a transcription factor, which will be discussed in subsequent sections. Since the NLS of YB-1 is noncanonical, whether this NLS interacts with importins needs to be examined. Experiments

inspecting the association between the noncanonical NLS of YB-1 and importins will not only validate the idea proposed above but also identify another type of NLS sequence recognized by importin α .

4.2.2 Ser102 and DNA Binding

Since CSD of YB-1 is known to bind to nucleic acids, we questioned whether Ser102 contributes to the ability of YB-1 to interact with the promoter of *EGFR*. We found that while Flag:YB-1 bound to the first 2 kb of the *EGFR* promoter, Flag:YB-1A102 was not able to interact with the first 1 kb promoter region. In addition, OSU-03012, which blocked YB-1 phosphorylation at Ser102 by Akt, prevented the interaction between YB-1 and the -1 kb of *EGFR* promoter. These data suggest that the binding of YB-1 to the first 1 kb of the *EGFR* promoter is dependent on the phosphorylation of Ser102 by Akt.

One may argue that disruption of Ser102 blocked nuclear translocation of YB-1 thus prevented the binding of YB-1 to the *EGFR* promoter. While this may be correct, we believe that Ser102 plays a role in YB-1:DNA interaction due to the following reasons. First, upon careful inspection, neither mutation of Ser102 to alanine nor treatment of OSU-03012 completely blocked nuclear shuttling of YB-1. In fact, there was still approximately 50% of YB-1 detected in the nucleus after disruption of Ser102 or OSU-03012 treatment. Second, abolishment of Ser102 almost fully prevented Flag:YB-1A102 to bind to the -1 kb of *EGFR* promoter. Similarly, the binding of YB-1 to the 1b region decreased to less than 50% upon OSU-03012 treatment. More intriguingly, OSU-03012 fully prevented the interaction between YB-1 and the 2a region of the *EGFR* promoter. Therefore, we suggest that in addition to nuclear translocation, Ser102 is also important for YB-1 to bind to DNA. Furthermore, the observation that YB-1 but not YB-1A102 bound to the -1 kb of *EGFR* promoter implies that this region may contribute to the regulation of

EGFR transcription by YB-1, which is discussed in section 4.3 below.

The presence of YB-1A102 in the nucleus explains the observation that YB-1A102 interacted with the 2b and 3 regions of the *EGFR* promoter. The ability of YB-1A102 to bind to these sites equally strongly as the wild type YB-1 indicates that the binding of YB-1 to the 2b and 3 regions is Ser102 independent and is not regulated via PI3K/Akt signaling. It is possible that YB-1 binds to this region solely due to the ability of YB-1 to recognize YREs containing the inverted CCAAT boxes without exerting any regulatory effect on *EGFR* expression. Alternatively, YB-1 may indirectly bind to this part of the promoter through another transcription factor, which then activates or repress *EGFR* transcription. Since the CSD of YB-1 is not involved in protein-protein interaction, phosphorylation at Ser102 by Akt may not be required for this complex formation and DNA binding. If this is true, then pathway(s), besides Pi3K/Akt signaling, that control the regulation of *EGFR* by YB-1 may exist.

4.3 Regulation of *EGFR* Gene Expression by YB-1

By using luciferase reporter assays we further examined whether YB-1 activates the -1 kb of *EGFR* promoter. Transfection of the YB-1 expression vector hardly induced the luciferase activity. In this experiment, two cell lines were used. The HEK293T cells were originally derived from embryonic kidney. It was shown that YB-1 is highly expressed in fetal tissues (Spitkovsky *et. al.* 1992), and indeed we found that HEK293T cells expressed high levels of YB-1. The level of pAkt in HEK293T was also observed to be elevated (Figure 3). Another cell line used in the luciferase reporter assays was the MDA-MB-468 cells, which also exhibit constitutively activated Akt signaling and express high level of *EGFR* (Figure 3). Therefore, it is possible that the *EGFR* promoter cannot be further activated by exogenous YB-1 in cell lines chosen for this study. Alternatively, since the region between -1 kb and -2 kb of the *EGFR* promoter contains

many YREs and was bound by YB-1, it is possible that these YREs may be required for optimal transcriptional activity of YB-1. The pER construct excluded all sites in the 2b and 3 regions of the *EGFR* promoter, and thus transcriptional activity could not be increased in cells co-transfected with YB-1.

Despite the inability to enhance *EGFR* transcription by exogenous YB-1, transfection of YB-1A102 expression vector significantly suppressed *EGFR* promoter activity, suggesting YB-1A102 may exert a dominant negative effect to interfere with the function of endogenous YB-1 in regulating *EGFR* transcription. This notion can be supported by the observation that YB-1A102 also interfered the nuclear translocation of the endogenous YB-1 (section 3.1.2). Although exogenous YB-1 could not activate *EGFR* transcription in HEK293T and MDA-MB-468 cells, we were able to show that expression of Flag:YB-1, but not Flag:YB-1A102, increased the level of EGFR protein and mRNA in MCF-7 cells. While the amount of mRNA in MCF-7 Flag:EV and MCF-7 Flag:YB-1A102 were comparable, the level of EGFR protein was further reduced in MCF-7 Flag:YB-1A102, suggesting that YB-1A102 may also have a dominant negative effect on protein translation. Collectively, these data indicated that YB-1 contributes to the regulation of *EGFR* gene expression. In a similar study, Berquin *et al.* recently illustrated in normal epithelial cells that transfection of YB-1 cDNA also increased EGFR mRNA and protein levels. However, promoter-reporter assays demonstrating a direct role of YB-1 in *EGFR* transcription were not performed in their study (Berquin *et al.* 2005).

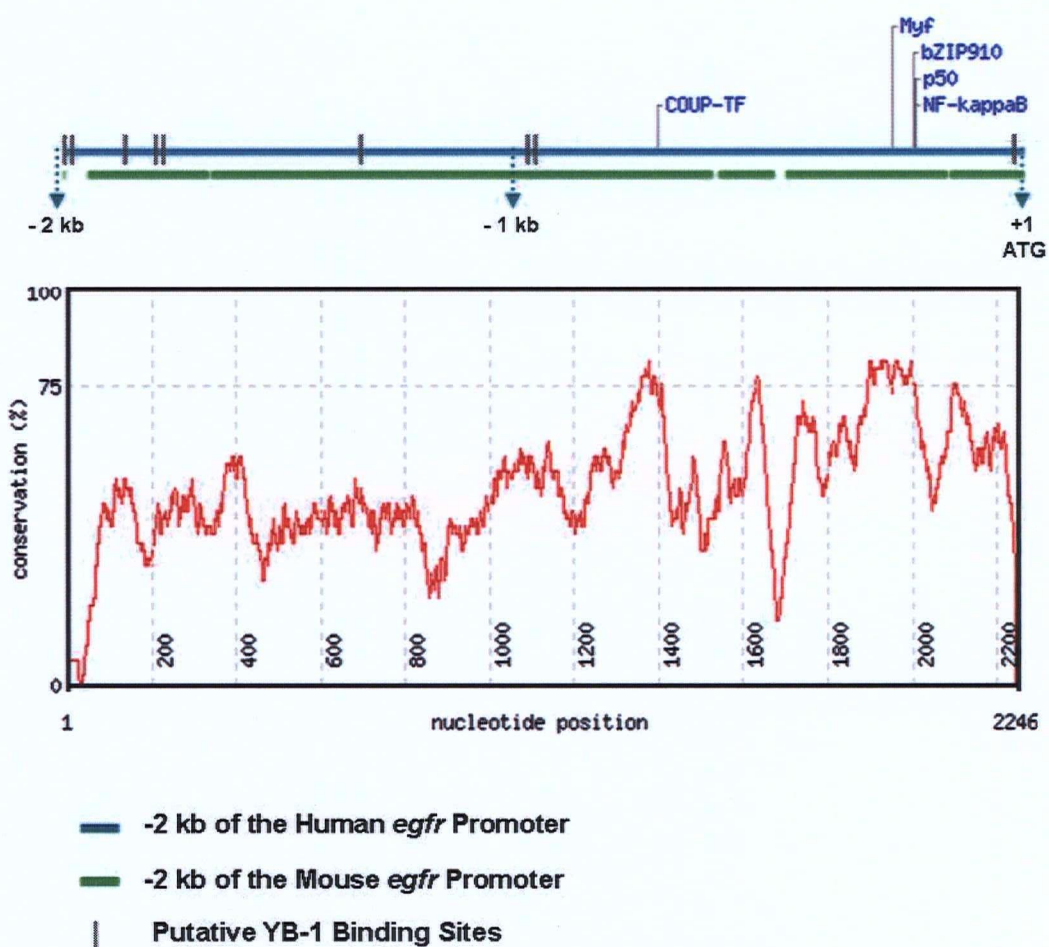
There are several ways in which YB-1 may regulate *EGFR* transcription. The most straightforward approach is to bind to the promoter of EGFR directly. In this study we demonstrated the interaction between YB-1 and *EGFR* promoter using ChIP; however, this method does not reveal whether the interaction is direct or is mediated by other co-activators. YB-1 is known to form complexes with other transcription factors including p53, RelA (p65),

activating protein-2 (AP-2), the multivalent zinc finger factor (CTCF), and YY1 (Okamoto *et. al.* 2000, Raj *et. al.* 1996, Mertens *et. al.* 2002, Chernukhin *et. al.* 2000, Li *et. al.* 1997). None of these YB-1 binding partners has been shown experimentally to regulate *EGFR* transcription in breast cancer cells. In addition, by examining the -2 kb of the *EGFR* promoter using CONSITE (<http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite>), we did not find any of the above YB-1 binding partners as potential transcription factors that regulate the first 2 kb of the *EGFR* promoter (Figure 14). YB-1 and other proteins that regulate *EGFR* transcription (section 1.4.1) were not identified as possible regulators for the *EGFR* promoter by CONSITE because these transcription factors were not included by CONSITE as candidate transcription factors.

Interestingly, NF- κ B and p50, both of which are members of the NF- κ B/rel protein family, were discovered to be possible regulators for the first 1 kb of the *EGFR* promoter by CONSITE (Figure 14). One member of the NF- κ B/rel protein is p65/RelA (Pikarsky and Ben-Neriah 2006). The p65/RelA protein is able to form heterodimers with p50 and is a subunit of the NF- κ B complex (Pikarsky and Ben-Neriah 2006). The NF- κ B complexes are regulated by Akt (Nicholson and Anderson 2002, Romashkova *et. al.* 1999, Kane *et. al.* 1999) and have been implicated in cancer including breast carcinoma (Bours *et. al.* 2000, Garg *et. al.* 2003, Zhou *et. al.* 2005, Wu and Kral 2005). Since YB-1 has been shown to interact with p65 to regulate the transcription of the JC virus (Raj *et. al.* 1996), it is possible that YB-1 induces *EGFR* transcription by interacting with the p65 subunit of the NF- κ B complex. If it is true, then the NF- κ B complex may be the protein that binds to the DNA while YB-1 is the co-activator to enhance transcription. Alternatively, YB-1 may be the protein that interacts with the inverted CCAAT boxes of *EGFR* whereas NF- κ B acts as the co-activator. Recently, our lab was able to demonstrate by EMSA that YB-1 binds directly to the 2 YREs in the 2a region of the first 1 kb of the *EGFR* promoter. Mutations of the inverted CCAAT boxes in one or both YREs inhibited

Figure 14
Potential Transcription Factors that Regulate the -2 kb of the *EGFR* Promoter Predicted by CONSITE

Several transcription factors, including NF- κ B, p50, bZIP910, Myf, and COUP-TF, were predicted as potential regulators of the *EGFR* promoter by CONSITE (<http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite>). The -2 kb of the *egfr* promoters of both human and mouse were retrieved from the USCS genome browser (<http://genome.ucsc.edu/>) and analyzed for all the transcription factors available in CONSITE. A 75% conservation between the human and mouse *EGFR* promoter and an 80% of transcription factor threshold were the criteria used to predict potential regulators of the *EGFR* promoter.



YB-1:DNA complex formation (unpublished data). Therefore, we argue that YB-1 binds directly to the -1 kb of the promoter to activate *EGFR* transcription. It would however be interesting to examine if NF- κ B plays a role in YB-1-mediated *EGFR* transcription activation. Finally, the C-terminal domain of YB-1 is known to facilitate protein-protein interaction or homo-multimerization (Tafari and Wolffe 1992, Swamynathan *et. al.* 1998, Nambiar *et. al.* 1998, Bouvet *et. al.* 1995); thus, the possibility that the binding of YB-1 to *EGFR* promoter is enhanced by YB-1 homo- or hetero-multimerization cannot be ruled out.

4.4 The Role of Akt, YB-1, and EGFR in Breast Cancer

The relationship between Akt and YB-1 in cancers has been demonstrated in several studies. It was recently shown that YB-1 but not YB-1A102 enhanced breast cancer cell growth, suggesting that Akt and YB-1 together exert a stimulatory effect on tumor growth (Sutherland *et. al.* 2005). This is in contrast to the study in which YB-1 suppresses Akt-mediated oncogenic transformation of chicken embryo fibroblasts by inhibiting protein synthesis (Bader *et. al.* 2003, 2005). However, this work advocating YB-1 as a tumor suppressor was contradicted by another study, which showed that YB-1 activates mRNA translation upon phosphorylation by Akt (Evdokimova *et. al.* 2006). We suspect that the discrepancy may be due to the different model systems used in the studies. In the context of breast cancer, much of evidence supports the oncogenic function of YB-1. For example, YB-1 is over-expressed in breast tumors, and over-expression of YB-1 is associated with poor prognosis (Bargou *et. al.* 1997, Rubinstein *et. al.* 2002, Janz *et. al.* 2002). The fact that pAkt is co-expressed with YB-1 (Sutherland *et. al.* 2005) further validates the idea that Akt and YB-1 work together to promote breast cancer growth. Since we have shown that the expression of EGFR by YB-1 is dependent on Ser102, it is considered that YB-1 stimulates breast cancer cell growth by up-regulating *EGFR* transcription.

This proposition is verified by our recent work demonstrating that knocking-down YB-1 using siRNA suppressed EGFR expression and breast cancer cell growth (Wu *et. al.* 2006). It will be beneficial to examine whether inhibiting EGFR would block YB-1-mediated tumor cell growth. In fact, it has been demonstrated that while transfection of YB-1 cDNA leads to increased EGFR in the membrane, antibody targeting EGFR decreased the growth of cells that are transfected with YB-1 cDNA (Berquin *et. al.* 2005). Taken together, we propose that upon PI3K/Akt activation, YB-1 promotes breast cancer cell growth in part via inducing EGFR expression.

It is now well recognized that there exist subtypes of breast cancer, namely the ER(+) luminal subtype A and B, the ER(-) normal breast like, ER(-) ERBB2+, and ER(-) basal like breast carcinoma (Perou *et. al.* 2000, Sorlie *et. al.* 2001, 2003). At least two studies have shown that the expression of EGFR is associated with the basal-like breast cancer (Nielson *et. al.* 2004, Livasy *et. al.* 2006). The question raised here is whether YB-1 also correlates with the basal like breast tumors. In a study conducted by van't Veer *et. al.* using tumor tissue microarrays, YB-1 was clustered in the basal-like subtype in a cohort of women who possessed BRCA-1 mutation (van't Veer *et. al.* 2002, Sorlie *et. al.* 2003). In another study, YB-1 falls into a novel unknown cluster (Sorlie *et. al.* 2001). One possible explanation for this contradiction may be that the validated basal-like markers, such as cytokeratin 5, are highly expressed in both normal breast tissue and basal-like breast cancer. However, YB-1 is only detected in breast tumor tissues. Thus, in this study YB-1 failed to cluster with markers of the basal-like breast carcinoma. Interestingly, upon closer inspection of their data it is evident that YB-1 is most intensely stained in tumors that fall into the basal-like subtype. A connection between YB-1, EGFR, and basal-like subtype has been implicated in the SUM149 cell line. The SUM149 cells display the gene expression profile representing the basal-like breast cancer. It was observed that both EGFR and YB-1 are highly expressed in these cells (manuscript in preparation, Habibi *et. al.* 2006). Equally

important, our lab also showed that YB-1 and EGFR are co-expressed in breast tumor tissues (Wu *et. al.* 2006). Given that YB-1 regulates EGFR expression (section 3.2), there is no reason why we should not assess the role of YB-1 in the basal-like breast carcinoma. Such investigation should definitely broaden our knowledge towards the basal-like disease that is difficult to manage with current treatments.

4.5 Targeting YB-1 in Breast Cancer

As activated Akt is associated with tumor aggressiveness and drug resistance, the components of the Akt signaling pathway have become attractive targets for developing new therapeutic interventions for breast cancers. Drugs such as trastuzumab that target the RTKs has been shown to be effective in some aggressive breast tumors. However, resistance to the RTK inhibitors has been observed in patients whose tumors are PTEN-negative. Therefore, it is of interest to look for small molecule inhibitors that target proteins that are more downstream of the PI3K/Akt pathway. Although many Akt downstream substrates have been found, YB-1 is an attractive therapeutic target because YB-1 is over-expressed in breast tumors but not in normal breast tissue. In addition, the importance of YB-1 in breast cancer has been established by our lab and others. Here, we have further demonstrated that activation of PI3K/Akt pathway activates YB-1, which up-regulates EGFR expression and promotes breast tumor cell growth. In this study, we examined the ability of a PDK-1 inhibitor, OSU-03012, in inhibiting the functions of YB-1. Another advantage of OSU-03012 is that since OSU-03012 inhibits PDK1, which is downstream of PTEN, this drug may have the potential to treat breast tumors that are PTEN-negative. We found that OSU-03012 was able to block Akt activity both in PTEN-positive MDA-MB-453 (Noh *et. al.* 2004) and PTEN-negative MDA-MD-468 cells (Nicholson *et. al.* 2003). Furthermore, OSU-03012 also prevented YB-1 nuclear translocation and *EGFR* promoter binding. This data

not only showed in principle that blocking YB-1 may be a novel approach to target EGFR over-expressing breast cancers, but also demonstrated the potential of OSU-03012 to be a therapeutic drug for PTEN-negative breast carcinoma. In addition to breast cancer, OSU-03012 has been studied in other cancers such as chronic lymphocytic leukemia (CLL) and pancreatic cancer (Johnson *et. al.* 2005, Li *et. al.* 2006). In both studies, OSU-03012 decreased CLL and pancreatic cancer cell growth.

Previously, our lab illustrated that OSU-03012 decreased the level of EGFR in mouse xenograft (unpublished data). However, OSU-03012 failed to reduce EGFR protein level in MDA-MB-468 in cell culture (data not shown). MDA-MB-468 was chosen for this *in vitro* drug study because it expresses high level of EGFR. The extremely high expression of EGFR in this cell line is partly due to gene amplification (Filmus *et. al.* 1985), but the region(s) of gene amplification is unknown. It is also possible that the expression caused by gene amplification may be too high to be reduced by OSU-03012 under the conditions studied. Alternatively, the amplified *EGFR* may harbor different transcription regulatory mechanism; thus, OSU-03012 which target PI3K/Akt and YB-1 may not be effective. Furthermore, as discussed previously OSU-03012 did not inhibit phosphorylation of YB-1 in MDA-MB-468 cells. Evidently, there is still much work to be performed to assess the efficacy of OSU-03012 in treating EGFR over-expressing breast cancer. Suppressing EGFR expression by OSU-03012 in other breast cancer cell lines is currently under investigation. This study will also provide insights into whether the mechanism causing EGFR over-expression is useful in selecting patients who may respond to OSU-03012 in future clinical trials.

4.6 Conclusion and Future Direction

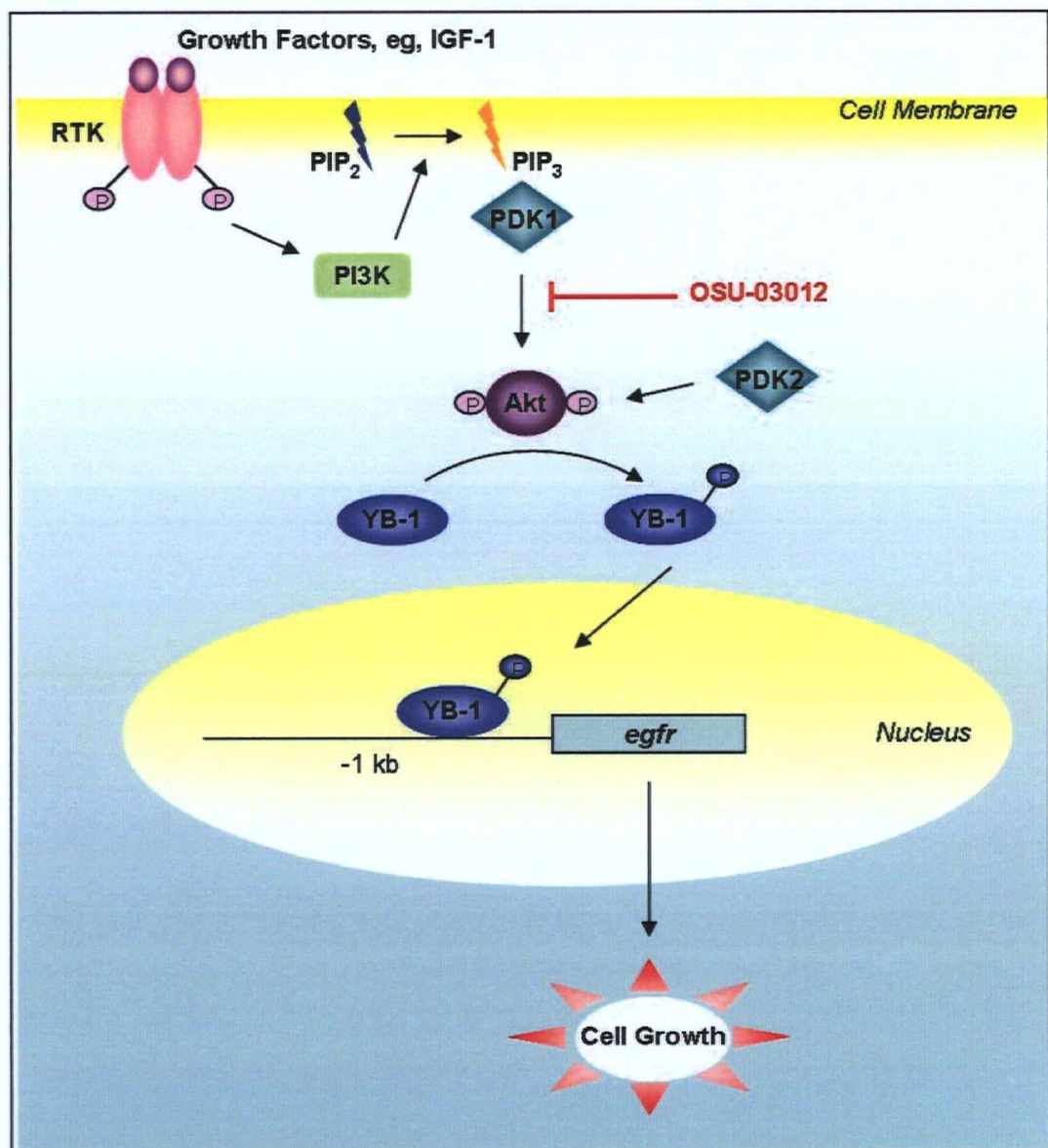
In conclusion, YB-1 is phosphorylated at Ser102 through the PI3K/Akt signaling pathway. The phosphorylation of Ser102 is important for YB-1 to translocate into the nucleus and to bind to the first 1 kb of the *EGFR* promoter. This leads to activation of *EGFR* transcription, resulting in increased level of mRNA and protein. The elevated expression of EGFR then can promote breast cancer cell growth (Figure 15).

This study has provided novel insights into how YB-1 is regulated and how YB-1 promotes breast cancer cell growth. Undoubtedly, many experiments are still required to further verify the model proposed here. First of all, we assessed Ser102 phosphorylation of YB-1 by immunoprecipitation and western blotting using a phospho-serine specific antibody. The disadvantage of this approach is that other phosphorylated serines are also detected. To specifically examine Ser102, our lab is currently developing a phospho-Ser102-YB-1 antibody. This antibody will also allow easier screening for small molecule inhibitors such as OSU-03012 that inhibits YB-1 activity.

Secondly, we have established the importance of Ser102 in YB-1 functioning by comparing the behaviors of YB-1 and the YB-1A102 mutant. To further validate the role of Ser102, one can generate an “activating” YB-1 mutant in which Ser102 is changed to glutamate or aspartic acid to mimic the negative charge of the phosphate group. The activating YB-1 mutant is expected to locate primarily in the nucleus and to bind to the promoter of *EGFR* more efficiently. Consequently, breast cancer cells expressing the activating mutant are anticipated to grow much faster. For the past few years, our lab has focused on the capability of YB-1 to enhance breast tumor cell growth. It is now time to extend this knowledge into animal models. For instance, by using an inducible system, one can investigate whether YB-1A102 or the activating YB-1 mutant decreases or increases the size of the tumors that are pre-formed in mice.

Figure 15
YB-1 Up-regulates EGFR Expression and Enhances Breast Cancer Cell Growth via the PI3K/Akt Signaling Cascade

Upon growth factor stimulation, the PI3K/Akt pathway is activated. YB-1 is then phosphorylated at Ser102 by Akt and translocates into the nucleus. Once in the nucleus, YB-1 binds to the first 1 kb of the *egfr* promoter and activates transcription. The Expression of EGFR is therefore induced, resulting in enhanced breast cancer cell growth.



Furthermore, since it has been shown that YB-1 promotes mammary tumor formation in nude mice, it would be interesting to examine whether YB-1A102 or the activating mutant exert any effect on tumorigenesis in small animals.

Thirdly, we have shown that YB-1 regulates the first 1 kb of the *EGFR* promoter. There are several YREs contained in this region; thus, we are interested in fine-mapping the YB-1 binding site(s). In fact, by using EMSA our lab has recently found that both YREs in the 2a region contribute to YB-1 binding. Luciferase assays using constructs with mutated YREs will further confirm the role of these YREs in *EGFR* transcription regulation by YB-1.

Finally, the ability of OSU-03012 to block YB-1 nuclear trafficking and DNA binding was illustrated. We are in the process to examine if OSU-03012 may also inhibit the binding of YB-1 to the 2b and 3 regions of the *EGFR* promoter. Since the interaction between YB-1 and these sites seems to be Ser102 independent, the inhibition of such interaction would further suggest the presence of other phosphorylation sites in YB-1 recognized by components of the PI3K pathway. Furthermore, whether OSU-03012 inhibits YB-1 phosphorylation and suppresses EGFR expression require further investigation. Our lab has previously investigated the efficacy of OSU-03012 in mice. Initial results revealed that OSU-03012 decreased tumor size, inhibited YB-1 to bind to the *EGFR* promoter, and suppressed EGFR expression. Thus, it is motivating to expand the study to further evaluate the potential of OSU-03012 as a therapeutic drug.

We have shown that YB-1 stimulates tumor cell growth by up-regulating EGFR expression. Since the expression of EGFR is associated with the basal-like breast carcinoma, it is of special value to assess whether YB-1 also plays a role in the basal-like subtype. It is envisioned that such a study will certainly advance the field of basal-like subtype, which we know very little about. More importantly, this investigation will allow us to further evaluate whether YB-1 serves as a potential target to treating the basal-like breast tumors. Currently more

than 10 genes have been identified to be regulated by YB-1. However, the relationship between YB-1 and most of them has not been determined in cancers. Our lab is interested in discovering novel YB-1 target genes in the context of cancers by using the ChIP-on-chip technology.

Moreover, since we are most interested in learning more about the basal-like breast cancer, it is valuable to inspect which genes are deregulated by YB-1 in this subtype of the disease. Finally, YB-1 is known to be over-expressed in a spectrum of malignancies including colon, lung, and ovarian cancers. However, how YB-1 contributes to these cancers is poorly understood. Since EGFR is also implicated in these tumors, the knowledge obtained from this study may be applied to other cancers and may be of value in treatment of other tumor types.

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APPENDIX**BUFFERS AND REAGENTS****Aves wash buffer A**

10 mM Tris buffer (pH 8.0)
0.02% sodium azide
0.01% BSA

150 mM NaCl
0.1% Triton X-100

Aves wash buffer B

10 mM Tris buffer (pH 8.0)
0.02% sodium azide

150 mM NaCl

Aves wash buffer C

50 mM Tris buffer (pH 6.8)

Borgatti Buffer B

10 mM Tris-HCL (pH 7.2)

2 mM MgCl₂

Buffer A

10 mM HEPES (pH 7.9)
10 mM KCl
0.1% NP-40

1.5 mM MgCl₂
1 mM EDTA

Buffer C

0.42 M NaCl
1.5 mM MgCl₂

20 mM HEPES (pH 7.9)
20% glycerol

ChIP dilution buffer

0.01% SDS
2 mM EDTA
150 mM NaCl

1.1% Triton X-100
16.7 mM Tris-HCl (pH 8.1)
ELB Buffer

6x DNA loading buffer

25 mg bromophenol blue
 5 ml glycerol
 make up to 10 ml with distilled water

25 mg xylene cyanol
 100 μ l of 1 M Tris-HCl (pH8.0)

Egg Lysis Buffer (ELB)

100 mM HEPES (pH 7.4)
 10 mM EDTA (pH 8.0)
 1 mM DTT

500 mM NaCl
 0.2% NP-40

High salt buffer

0.1% SDS
 2 mM EDTA
 500 mM NaCl

1% Triton X-100
 20 mM Tris-HCl (pH 8.1)

LiCl buffer

0.25 mM LiCl
 1% deoxycholate
 10 mM Tris-HCl (pH 8.0)

1% IGEPAL
 1 mM EDTA

Low salt buffer

0.1% SDS
 2 mM EDTA
 150 mM NaCl

1% Triton X-100
 20 mM Tris-HCl (pH 8.1)

RIPA buffer

150mM NaCl
 0.5% sodium deoxycholate
 50mM Tris (pH7.2)
 adjust pH to 8.0

1% NP-40
 0.1% SDS

Phosphate buffered saline (PBS)

8 g NaCl
 1.44 g Na_2HPO_4
 make up to 1 L with distilled water
 adjust pH to 7.4

0.2 g KCl
 0.24 g KH_2PO_4

Protease Inhibitor Cocktail (PIC)

2.5mM sodium pyrophosphate	1mM β -glycerol phosphate
1mM sodium vanadate	1 μ g/ml leupeptin
1mM PMSF	1mM NaF
1 μ g/ml aprotinin	

Sample loading buffer

0.5 M Tris-HCl (pH 6.8)	0.8 ml glycerol
10% SDS	1% bromophenol blue
3.8 ml distilled water	
add 0.4 ml of 14.3 M 2- β -mercaptoethanol before use	

SDS lysis buffer

1% SDS	10 mM EDTA
50 mM Tris-HCl (pH 8.1)	

3% Stacking gel for SDS-PAGE

4% acrylamide/bis	0.125 M Tris (pH 6.8)
0.1% SDS	0.05% ammonium persulfate
0.1% TEMED	

6% Separating gel for SDS-PAGE

6% acrylamide/bis	0.375 M Tris (pH 8.8)
0.1% SDS	0.05% ammonium persulfate
0.1% TEMED	

12% Separating gel for SDS-PAGE

12% acrylamide/bis	0.375 M Tris (pH 8.8)
0.1% SDS	0.05% ammonium persulfate
0.1% TEMED	

10x Transfer buffer

140 g glycine	30.3 g Tris-base
To make 1x transfer buffer containing 20% methanol, dilute 100 ml of 10x transfer buffer in 700 ml distilled water and 200 ml of methanol. Chill before use.	

TAE buffer

242 g Tris-base

57.1 ml acetic acid

100 ml of 0.5 M EDTA

make up to 1 L with distilled water

adjust pH to 8.5

TE buffer

1mM EDTA

10 mM Tris-HCl (pH 8.0)